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ABSTRACT

This laboratory manual presents information and techniques dealing with aquatic microbiology as it relates to environmental health science, sanitary engineering, and environmental microbiology. The contents are divided into three categories: (1) ecological and physiological considerations; (2) public health aspects; and (3) microbiology of water and wastewater treatment. Each category contains a series of experiments pertinent to that area of study. These experiments outline the purpose, general references, introductory information, apparatus, and reagents. Schematics are provided for comparison of data collected as an aid for data analysis. (CS)

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AQUATIC MICROBIOLOGY LABORATORY MANUAL

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Association of Environmental Engineering Professors

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AQUATIC MICROBIOLOGY

LABORATORY MANUAL

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November 1976

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TABLE OF CONTENTS

I. ECOLOGICAL AND PHYSIOLOGICAL CONSIDERATIONS

A. 1. Representative Aquatic Microbial Life

2. Isolation, Observation, Enumeration of Aquatic Bacteria

3. The Fungi

4. The Protozoa

5. The Algae

B. 1. The Effect of Nutritional and Environmental Factors on Growth

2. The Measurement of Algal Growth Potential Using Batch Cultures

C. 1. Dissimilatory Sulfate Reduction

2. Oxidation of Reduced Sulfur Compounds

3. The Winogradsky Column

D. 1. Nitrogen Fixation

2. Ammonification and Nitrification

3. Denitrification

E. 1. Survival of Non-Indigenous Bacteria in Natural Waters

F. 1. Measurement of Primary Productivity

G. 1. The Growth of Microorganisms on Surfaces

II. PUBLIC HEALTH ASPECTS

A. 1. Enteric Bacterial Pathogens; Isolation of *Salmonella* and *Shigella* from Sewage

B. 1. Indicator Organisms - Enumeration of Coliforms and Fecal Coliforms by the Most Probable Number Method

2. Indicator Organisms - Enumeration of Fecal Streptococci by the Most Probable Number Method

3. Enumeration of Indicator Organisms by the Membrane Filter Technique

CONTENTS (continued)

III. MICROBIOLOGY OF WATER AND WASTEWATER TREATMENT

A. 1. The Microbial Community in Activated Sludge

B. 1. Kinetic Models for the Continuous Culture of Microorganisms

B. 1. A. Determination of the Michaelis-Menten Constant and Maximum Reaction Rate of Urease

b. Determination of Kinetic Constants for Activated Sludge

2. Batch Determination of Cell Yield in Aerobic Systems

C. 1. Biodegradability Introduction

2. Biodegradability of Butyl Alcohols

3. Biodegradation of Alkyl Aryl Sulfonates

4. Adaptation of Mixed Cultures - The Bio-Oxidation of Mono- and Di-Saccharides

D. 1. Toxicity of Heavy Metals to Mixed Microbial Cultures

E. 1. Removal of Bacteria and Bacteriophage by Rapid Sand Filtration

2. Removal of Bacteria by Water Chlorination

APPENDIX I. CARE AND USE OF MICROSCOPE

I. ECOLOGICAL AND PHYSIOLOGICAL CONSIDERATIONS

I-A-I REPRESENTATIVE AQUATIC MICROBIAL LIFE

INTRODUCTION: CLASSIFICATION OF MICROORGANISMS

The biologist has historically been involved in the classification of life forms. Much effort has been expended to develop philogenetic classifications that relate the various forms of life on the basis of their social history or evolutionary development. Traditionally, life forms have been placed into either the plant or animal kingdoms and then further subdivided into phylum, class, order, family, genus, species and variety. With most animals and plants, this classification has been quite successful. Difficulties arise, however, when attempts are made to place microorganisms into these two kingdoms and to determine their philogenetic interrelationship. This can be illustrated by the Euglena. This single-cell creature is photosynthetic (plant-like), yet has many "animal qualities" such as no rigid cell wall and motility. Because of such difficulties in the classification of microorganisms, a third group or "kingdom" called the Protista has been introduced which includes the majority of microorganisms.

The Protista are commonly subdivided into the higher Protista which include green algae, protozoa, and fungi and the lower Protista which include blue-green algae and bacteria.

The separation of the Protista into two major subgroups is based on certain characteristic cellular structures of each group. The higher Protista have eucaryotic cells while the cells of the lower Protista are procaryotic. Major differences between these two cell types are listed in Table I-A-I.

This classification scheme is not all-inclusive because some microorganisms, in particular the viruses, do not have any of the characteristics listed in Table I-A-I. They are frequently included as lower Protista as a convenience. For more detailed information the student is referred to modern texts on microbiology. In practice, the Protista are assigned to their various subgroups principally on the basis of individual and group cellular morphology, on natural and laboratory growth characteristics, when appropriate on their life cycle (e.g. sexual phase spore development); on their biochemical (enzymatic) diversity, particularly for bacteria; and, on their immunological characteristics, particularly for viruses and certain bacteria.

TABLE I-A-I. SIGNIFICANT CHARACTERISTICS OF PROCARYOTIC AND EUCARYOTIC CELLS

Characteristic	Cell Type	
	Procaryotic	Eucaryotic
Nuclear Membrane	Absent	Present
Mitotic Division	Absent	Present
Chromosome Arrangement	Single	Multiple
Mitochondria	Absent	Present
Flagella (when present)	Single Strand	Multiple Strand

Often, the microbial ecologist will classify groups of microorganisms on the basis of their ecological activity rather than individual characteristics. Thus, numbers of relatively unrelated species can be grouped on the basis of their activity in cycling various elements (e.g. nitrogen and sulfur in the environment).

EXPERIMENT I-A-2

ISOLATION, OBSERVATION AND ENUMERATION OF AQUATIC BACTERIA

Purpose

To isolate, observe and identify bacteria isolated from the aquatic environment.

References

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4. Shewan, J.M. "The Differentiation of Certain Genera of Gram-Negative Bacteria Frequently Encountered in Marine Environments" in Symposiom on Marine Microbiology, C.H. Oppenheimer, ed., 1963.
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Introduction

Bacteria are classified as lower Protista. They are microscopic unicellular organisms with a variety of cell shapes which can be generally described as cocci (spheres averaging approximately 0.5 μm diameter) in various arrangements such as pairs, chains or clusters; as rod forms

which are longer than wide and in the size range 1.6-4.0 μm x 0.5-0.8 μm; and as spiral forms. The rods can be further described by the shape of the ends of the cell (e.g. blunt, club, fusiform) and by the arrangement of the cells (e.g. single, in chains, palisades). The presence or absence of endospores is also a morphological feature for rod-shaped organisms since a number form spores. The spiral-shaped bacteria may be short or comma-shaped organisms (vibrio) or a variety of longer spiral forms, some of which are very large (spirillum forms) and some very fine (the spirochaetes). The source of these organisms in fresh water is primarily from the surrounding soil or extraneous sources such as sewage. Thus, most of the bacteria encountered are allochthonous (from other sources than water) to water and autochthonous (native) to soil. Even though most of the bacteria in waters originate from the soil, the water environment is sufficiently different from soil that there is usually a different frequency distribution of bacterial types in the two environments. Thus, waters will usually have a predominance of gram negative bacteria while soils will frequently have more gram positive than gram negative organisms. (In the marine environment or in salt lakes, one is apt to find bacteria which are autochthonous to that environment.)

The distribution of bacteria in aquatic environments such as lakes and ponds will also vary spatially. Usually only a small fraction of the total bacteria are freely suspended in the water column; greater numbers are found attached to suspended particles (episamic) and even greater numbers are associated with bottom sediments.

To determine the types of bacteria present in water, the microbiologist normally places a small amount of water into a nutrient medium

and allows the microorganisms present to grow to such numbers that they can be more readily seen and identified. The types of microorganisms isolated will depend not only on the original population present in the water but also on the type of growth medium and conditions of incubation. Thus, when bacterial numbers and types isolated from a water source are reported, it is important to report the growth conditions. To illustrate this point one need only compare the species isolated by growing bacteria on a solid nutrient medium with those that develop on microscope slides immersed in water for a few days.

Bacteria are normally isolated by growing them on a solid nutrient medium, usually with an agar base. Cells will be trapped in or on the solid medium and will grow into colonies which are usually visible to the naked eye. Isolated colonies are selected, resuspended and grown again on the solid medium until it is certain that the colonies formed are produced by one kind of bacterium. Microorganisms from such a colony are considered to be in pure culture. Identification of bacteria is normally based upon the characteristics of a pure culture.

There are numerous schemes used for bacterial identification which vary widely in their complexity depending upon the degree of refinement required. Frequently, simple schemes are developed so that isolated bacteria can be classified into relatively large but convenient groupings. Identification may include a morphological description of the individual cell and groups of cells; growth characteristics (e.g. optimum temperature, pH, redox environment and nutrients required for growth); biochemical diversity, (e.g. the ability of the isolate to produce acid and gas in the presence of various carbohydrates); and immunological characteristics.

The number of bacteria present in a given volume of water is generally determined using a colony counting method. A known volume of water is thoroughly mixed with a selected nutrient medium containing agar in a petri dish. The material is allowed to solidify and is then incubated at an appropriate temperature. Individuals or clumps of bacteria trapped in the solidified agar will grow and form colonies within a matter of hours or days. The colonies produced are counted and their number is taken as representative of the number of bacteria present in the volume of water sampled. If the number of colonies on a petri plate is greater than 300, counting becomes very difficult and dilution, usually ten-fold, is necessary.

Other methods of enumeration, such as direct microscopic counting and the use of turbidity as a measure of bacterial density are usually only useful when bacterial numbers are far greater than those encountered in natural waters.

Procedure

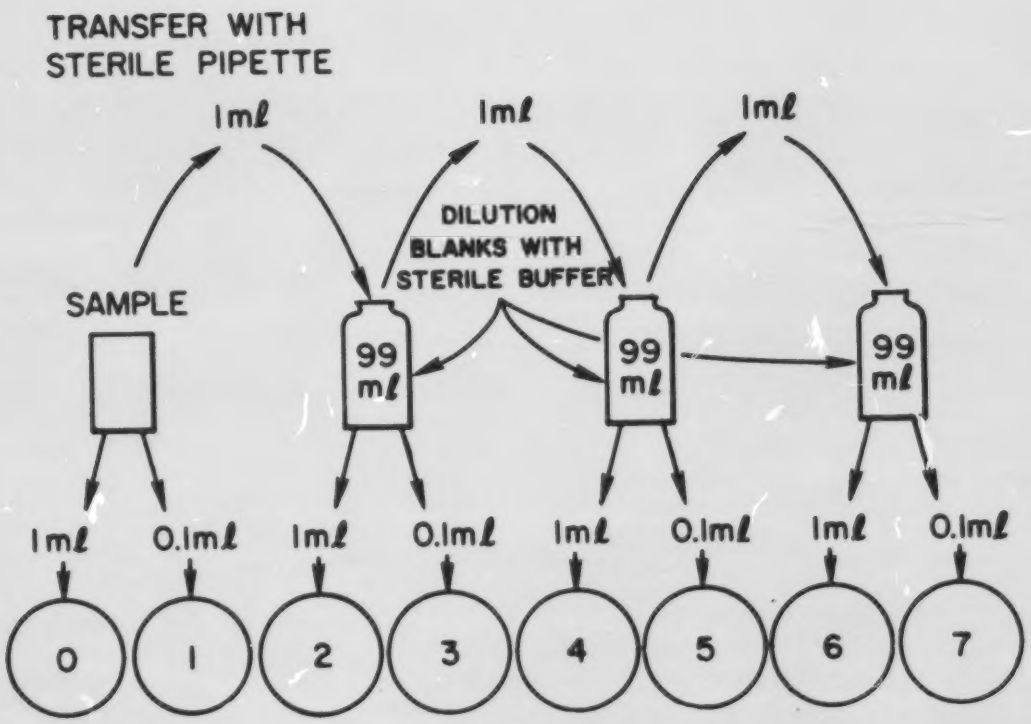
1. This experiment may be performed as a class exercise with each student being responsible for at least one type of sample.
2. Collect water samples (>50 mℓ) and sediment samples (10-15g wet weight) aseptically in sterile bottles from a local lake or pond.
3. Plating of Water Sample: Shake water sample thoroughly and pipette duplicate 1 mℓ and 0.1 mℓ portions into separate sterile petri dishes. Immediately add approximately 15 mℓ of melted nutrient agar, cooled to 45 C, and mix completely in the covered petri dish

using a figure-eight motion. Allow the agar to solidify then incubate the agar plates upside-down at 35 C for 24 to 48 hr.

4. Plating of Water Sample with Suspended Solids Removed: Centrifuge 15-40 mℓ water sample in a conical, graduated centrifuge tube at approximately 150-650 x G for 15 min to remove suspended solids and associated bacteria. This centrifugation will not significantly remove the freely suspended bacteria. Carefully decant the supernatant and note the volume remaining in the centrifuge tube. Resuspend the centrifuged material in 5-10 mℓ sterile, buffered water containing 1 mg/ℓ "Tween 80" (a nonionic wetting agent) and shake well. Plate this material in petri dishes and incubate as described in step 3.

5. Plating of Sediment Samples: To a sterile 250 mℓ erlenmeyer flask containing 100 mℓ buffered water plus 1 mg/ℓ "Tween 80", aseptically add a known weight of collected sediment (10-15 g wet weight) then thoroughly agitate the incubation flask for 10-15 min, preferably on a mechanical shaker. Use this suspended material to inoculate petri dishes as described previously. In some instances more dilution of the sample is required in order to arrive at 30-300 colonies per petri dish. If further dilutions are required, perform them using the scheme shown in Figure I-A-II.

6. Counting of Colonies: After 24 to 48 hr incubation, count the number of colonies on each plate, preferably those plates showing between 30-300 colonies being careful not to contaminate the petri plate with extraneous microorganisms. Some type of magnifying device such as a Quebec colony counter should be used for counting.



TO OBTAIN NUMBERS OF BACTERIA IN ORIGINAL SAMPLE MULTIPLY COLONIES BY:

10^0	10^1	10^2	10^3	10^4	10^5	10^6	10^7

FIGURE I-A-II. SCHEMATIC FOR PERFORMING TEN-FOLD DILUTIONS

Record the total number of colonies from each sample. Observe the size, color and shape of the colonies.

7. Preparation of Isolates: Select a random number of well-isolated colonies (5-10 percent of the total number) from a plate having less than 300 colonies. Using an inoculating wire, aseptically transfer material from these isolated isolated colonies to nutrient agar slants and incubate at 35 C for 24 hr.

   Note: In very careful work, selected isolates would be streaked out on to another agar plate and re-isolated at least once or twice before proceeding to this step. This step is omitted from this experiment because of time constraints.

8. Characterization of Isolates: The bacteria isolated in step 7 will now be characterized: a) gram stain; b) presence of endospores; c) motility; and, d) biochemical activities.

   a. Gram Stain: This is the most important bacteriological staining procedure. The manner in which isolated bacterial cells stain when using this staining schedule is associated with their cellular chemistry and is used to divide the bacteria into two great classes, those which are gram negative and those which are gram positive. This procedure should be performed on 24 hr cultures. All of the isolates should be gram stained.

      (i) Preparation of smear: Carefully clean a slide with tissue paper. Place a small drop of distilled or tap water on to a slide using a sterilized loop. With sterilized loop, lightly touch the growth which is to be examined and transfer a very small quantity of it to the

drop of water (just enough to make the drop of water very slightly cloudy). Spread the drop over a small area of the slide using the loop. (If the drop of water will not spread in an even thin film, the slide is not clean and is valueless for preparing a good mount). Air dry and then heat-fix the slide by passing the dried slide, film side up, three times through the flame of a Bunsen burner. Do not overheat. The film is now ready for staining.

(ii) Staining schedule (Huckers modification):

- Cover the fixed films for 1 min. with Crystal Violet ammonium oxalate solution.
- Wash in tap water for not more than 2 sec.
- Cover for 1 min with Gram's Iodine solution.
- Wash in tap water and blot dry.
- Decolorize for 30 sec. by gentle agitation in 95% ethyl alcohol and blot dry.
- Counterstain 10 sec. with Safranin solution.
- Wash in tap water and blot dry.

(iii) Microscopic observation: Place the stained slide on the microscope stage (smear side up). Using immersion oil and the oil immersion objective, examine the slide (see Appendix 1 on care and use of the microscope). Observe and record the shape and arrangement of the bacteria (e.g. rods, cocci) and their color. Bacteria which stain purple or blue are called gram positive; those which stain red are gram negative.

b. Presence of endospores: To determine the presence of endospores in gram positive rods, a differential staining technique is commonly used in which the vegetative cell stains one color while the endospore stains a contrasting color. Frequently, older cultures are used (48 hr - 7 days) to allow time for sporulation to occur. All gram positive isolates should be spore stained. It will be assumed that none of the gram negative isolates are spore-formers.

(i) Spore staining procedure (Bartholomew and Mittler "Cold" Method):

- Heat fix smear as for Gram stain.
- Stain 10 min with saturated aqueous Malachite Green solution without heating.
- Rinse with tap water for about 10 sec.
- Stain 15 sec in aqueous Safranin solution.
- Rinse, blot and dry.

(ii) Microscopic observation: Microscopically observe the stained material as described for the gram staining procedure. Examine the slide for bacteria in which most of the cell is stained red. The endospores will usually appear as round green inclusions or perhaps as green spheres free of cells. Observe and record shape and position of the spore in the vegetative cell.

c. Motility: Many bacteria can move using flagella which are usually too small to be seen with a light microscope except when very special staining procedures are used. In this

exercise bacterial motility will be observed in a liquid using the "hanging drop" method. Since many organisms lose their motility with age, it is important that young (24 hr) cultures be examined. All isolates should be examined for motility.

(i) Preparation for "hanging drop" motility test: Carefully clean a well slide (a slide that has a shallow concavity in the center) and place it on a clean place on the bench well side (depression-side) up. Carefully clean a No. 1 square coverglass with a piece of tissue paper. Apply a small dab of vaseline at each corner of the coverglass using a wooden applicator. Using a small loop, aseptically transfer a very small loopful of material from a liquid suspension to the center of the coverglass and then re-sterilize the loop. Pick up the well slide and carefully invert it over the coverglass, making sure that the loopful (drop) is in the center of the well. Press gently until the coverglass adheres to the well slide. Quickly invert the slide so the coverglass is facing up. The drop must remain suspended from the coverglass and must not touch the bottom of the well.

(ii) Examination of preparation for motility: Place the preparation on the microscope stage with the drop over the center of the condenser. Focus under low power on the edge of the coverglass. Move the slide across and look for the drop. When the drop has been located apply immersion oil to the slide, switch to the oil immersion

objective and examine the bacteria in the drop. A motile organism will quickly alter its position and be seen to move some distance in contrast to movement caused by Brownian motion whereby movements occur over very small distances and in a random fashion.

d. Biochemical Activities: Biochemical diversity in bacteria can be very extensive. In this exercise, three sample biochemical tests used to divide heterotrophic bacteria into a generalized taxonomic scheme will be studied. Each of the bacterial isolates from step 7 should be tested.

(i) Kovac's Oxidase Test: This test indicates the production of an enzyme that oxidizes a substrate by removing hydrogen and combining it with molecular oxygen. Test should be performed on organisms that have previously grown on nutrient agar for 24-48 hr. Remove some of the colony with an inoculating wire and smear in a 3-6 cm line on a piece of Whatman No. 1 filter paper to which 2-3 drops of a 1% solution of tetramethylparaphenylene diamine dihydrochloride in distilled water has been previously added. A positive reaction is indicated when the streak turns pink then maroon or black. No color change takes place if the enzyme is not produced by the isolate.

(ii) Arginine Broth: This test detects the hydrolysis of the amino acid arginine. Inoculate arginine broth with a 24-48 hr culture and incubate at room temperature for 24-48 hr. Add a few drops of Nessler's reagent. The formation

of a brown color shows the presence of ammonia which indicates that the isolate is capable of deaminating arginine.

(iii) Huge-Leifson Test (H-L Test): Some microorganisms ("oxidative") can metabolize glucose only when oxygen is the terminal electron acceptor (aerobic conditions). Other microorganisms ("fermentative") can metabolize glucose with terminal electron acceptors other than oxygen (i.e. under either aerobic or anaerobic conditions). This test distinguishes between these types of microorganism. Inoculate two tubes of H-L medium with material from a 24-hr agar slant culture. Cover the surface of one tube with sterile mineral oil. Incubate for 24-48 hr. Fermentative organisms produce acid (as indicated by a change in color of the indicator from blue to yellow in the medium) throughout both tubes. Oxidative organisms produce acid only in the aerobic (uncovered) tube. In the latter, acid appears first at the surface and then progressively moves toward the bottom of the tube. Slow oxidative reactions are sometimes preceded by a slight alkaline reaction indicated by a dark blue color. Some organisms give an alkaline reaction or no reaction.

Data Analysis and Questions

1. Record the results obtained from the sample(s) in a tabular form. Where appropriate use pooled data collected by the whole group.

2. Determine the total number of bacteria present in the water, those associated with suspended solids and those associated with the sediment.

3. Using pooled data, determine the standard deviation of the plate counting method by an examination of the variation in counts obtained from the same sample.

4. Determine the proportion of gram negative and gram positive bacteria found in the water and in the sediment samples. Do your results agree with the usually reported relationship between gram positive and negative bacteria in soil and water?

5. Using the data you have collected concerning staining, motility, and biochemical reactions, place each of your isolates into one of the generalized taxonomic categories shown in Figure I-A-III. Record the frequency of occurrence of the various types. Is there any relationship between frequency of bacterial type and sample source? Were any Clostridium types isolated? If not, why not?

Apparatus

Erlenmeyer flasks, 250 mℓ

Standard petri dishes

Centrifuge tubes, 50 mℓ

Dilution bottles, 99 mℓ

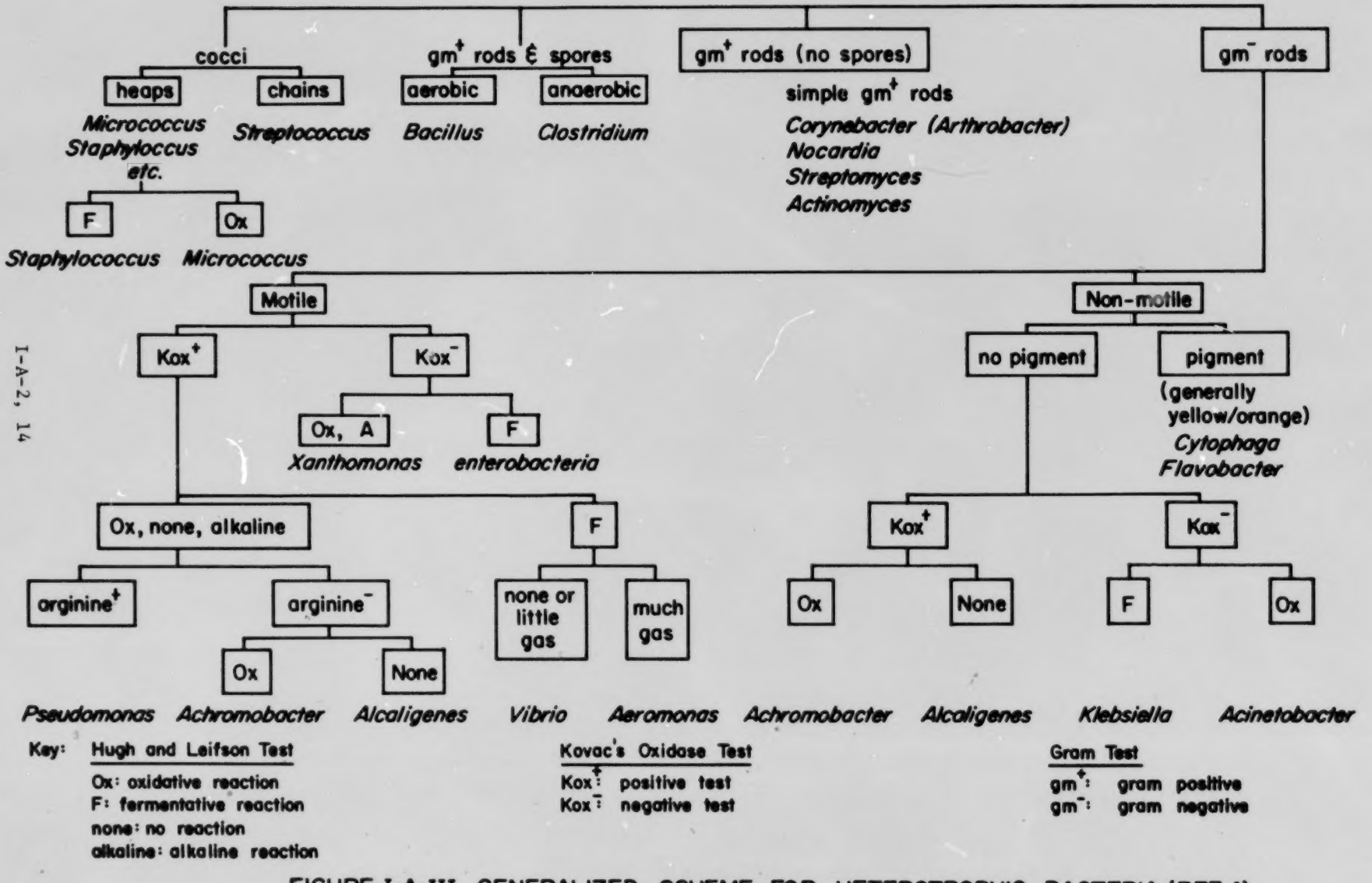
Microscope slides, plain

Well depression slides

Cover slips

Microscope, oil immersion

Incubators, 35 C and 20 C



Key: Hugh and Leifson Test
Ox: oxidative reaction
F: fermentative reaction
none: no reaction
alkaline: alkaline reaction

Kovac's Oxidase Test
Kox^+: positive test
Kox^-: negative test

Gram Test
gm^+: gram positive
gm^-: gram negative

FIGURE I-A-III. GENERALIZED SCHEME FOR HETEROTROPHIC BACTERIA (REF. 4)

Reagents

Gram Stain (Hucker's Modification):

Ammonium oxalate Crystal Violet: Mix 20 mℓ of a solution containing 2 g Crystal Violet in 20 mℓ 95% ethanol with 80 mℓ of a solution containing 0.8 g ammonium oxalate in 80 mℓ distilled water.

Gram's Iodine: Dissolve 1 g I_2 crystals and 2 g KI in 300 mℓ distilled water.

Decolorizing solution: 95% ethanol

Counter stain: Mix 10 mℓ 2.5% Safranin in 95% ethanol with 100 mℓ distilled water.

Spore stain (Bartholomew and Mittler):

Malachite Green solution: Saturated aqueous solution (approximately 7.6% w/v).

Safranin solution: 0.25% w/v aqueous solution.

Nessler Reagent: Nessler compound, Paragon (Paragon C & C Co., Inc., New York, N.Y.).

Tetramethylparaphenylene diamine dihydrochloride solution (TMPDH):

Dissolve 1 g TMPDH in 100 mℓ distilled water.

Buffered water: Dissolve 34 g KH_2PO_4 in 500 mℓ distilled water; adjust to pH 7.2 with 1N NaOH and dilute to 1000 mℓ. Add 1.25 mℓ stock buffer per 100 mℓ of distilled water; dispense into flasks in appropriate amounts and autoclave at 121 C for 15 min.

"Tween 80" (Polyoxyethylene sorbitan monolaurate): Difco.

Nutrient agar: Difco, in slants and tubes.

Huge-Liefson Broth:

Peptone	2 g
NaCl	5 g
K_2HPO_4	0.3 g
Agar	3 g
Bromthymol Blue, 1% w/v aqueous solution	3 mℓ
Distilled water	1000 mℓ

Dissolve ingredients and if necessary adjust pH to 7.1. Sterilize at 121 C for 20 min. Prepare a 10% aqueous solution of dextrose and sterilize by filtration. Aseptically add 10 mℓ to every 100 mℓ of the sterile, heated medium and dispense in 5 mℓ amounts in sterile 150 x 13 mm tubes. Prior to use heat in boiling water for 10 min to drive off any dissolved air present. Cool before use.

Arginine Broth:

Tryptone	5 g
Yeast extract	5 g
K_2HPO_4	2 g
L-arginine	3 g
Dextrose	0.5 g
Distilled water	1000 mℓ

Dissolve ingredients by heat and adjust the pH to 7.0. Add to tubes in 5-10 mℓ amounts and autoclave at 115 C for 10 min.

EXPERIMENT I-A-3

THE FUNGI

Purpose

To introduce the fungi with special reference to the common aquatic species, Saprolegnia.

References

1. Alexopoulos, C.J. Introductory Mycology. 2nd ed. John Wiley and Sons, New York, 1952.

2. Sparrow, F.K. Aquatic Phycomycetes, Exclusive of the Saprolegniaceae. University of Michigan Press, Ann Arbor, Michigan, 1943.

Introduction

The fungi are eucaryotic or higher Protista. They have many plant-like characteristics but lack chlorophyll. Most species occurring in nature are heterotrophic soil saprophytes. Many fungi are important plant and animal parasites. A number of fungi are typically aquatic.

While the commonly-conceived fungal form is a branching filament (hypha) which form a mass (mycelium) there are some fungal species that are not filamentous (e.g. the true yeasts and certain primitive aquatic species). Hypha may be septate, (cross-walls divide the filament) or non-septate (the filament is not divided into obvious cells). The fungi can be conveniently placed into four taxonomic categories:

Class I. Phycomycetes: The mycelium, when present, is non-septate. There are many nuclei in a single hyphal filament. These fungi reproduce

asexually both by simple multiplication of hyphae and by the production of spores contained in a sac-like structure called the sporangium. In some species (particularly aquatic forms) these sporangiospores are motile. If they have one or more flagella they are called zoospores. Sexual reproduction is accomplished through the production of gametes, typically contained in a gametangium, that fuse to form a zygote which will eventually germinate and produce new hyphae. To the aquatic microbiologist the phycomycetes are by far the most important and commonly encountered class of fungi.

Class II. Ascomycetes: The mycelium is septate and the cells are usually uni- or bi-nucleate. Asexual spores commonly called conidiospores are typically not contained in a special sac-like structure (exogenous). Sexual reproduction results in the production of ascospores contained in a special structure called the ascus. A typical member of this group is the common mold Penicillium.

Class III. Basidiomycetes: These are a group of fungi which include the familiar mushrooms, toadstools and shelf fungi. Exogenous asexual spores, if produced, are borne on condiophores or by fragmentation of the hypha into its component cells (oidium). Sexual reproduction results in the production of special spores borne on a structure called the basidium. The cap of a mushroom covers a multitude of basidia with their basidospores.

Class IV. Deuteromycetes (fungi imperfecti): These are a provisional taxonomic group containing those forms of fungi for which a sexual stage is unknown. Hyphae are typically all septate and reproduction is by conidia or other forms of asexual spores.

Procedure

1. Saprolegnia is a genus of common aquatic fungi frequently seen growing on fish, dead insects and other forms of organic matter. This genus is a readily-isolated and easily-maintained aquatic phycomycete which readily produces all stages of its life cycle.
2. A culture of Saprolegnia will be supplied by the instructor. If a culture is not readily available one can be generated quite simply by catching a house fly and floating the dead fly in a dish of pond water. Set this aside at room temperature for about 5 days. Usually Saprolegnia or its close relative Achlya sp. will appear as a filamentous growth radiating from the dead fly.
3. When the culture shows adequate growth prepare a wet mount of portions of the fungi and examine microscopically. Place a drop or two of Lactophenol Cotton Blue (Amann's solution) on a glass slide. Tease out a small piece of mycelium from the culture with a wire needle and place in the Lactophenol Cotton Blue. The mycelium can be pulled apart with two teasing needles. Cover the preparation with a coverslip and examine microscopically, first under low power and then under high power ("high dry") for fine details. The slide preparation may be kept for some time if the edges of the coverslip are sealed with clear nail polish.
4. Figure I-A-IV is a diagram of the life cycle of Saprolegnia sp. Compare your observations with the drawing. Identify the following: the somatic hyphae; sporangia containing sporangiospores (sporangiospores free of their sporangia may be seen since many of these will have been forced free when the cover slip was put in place); the

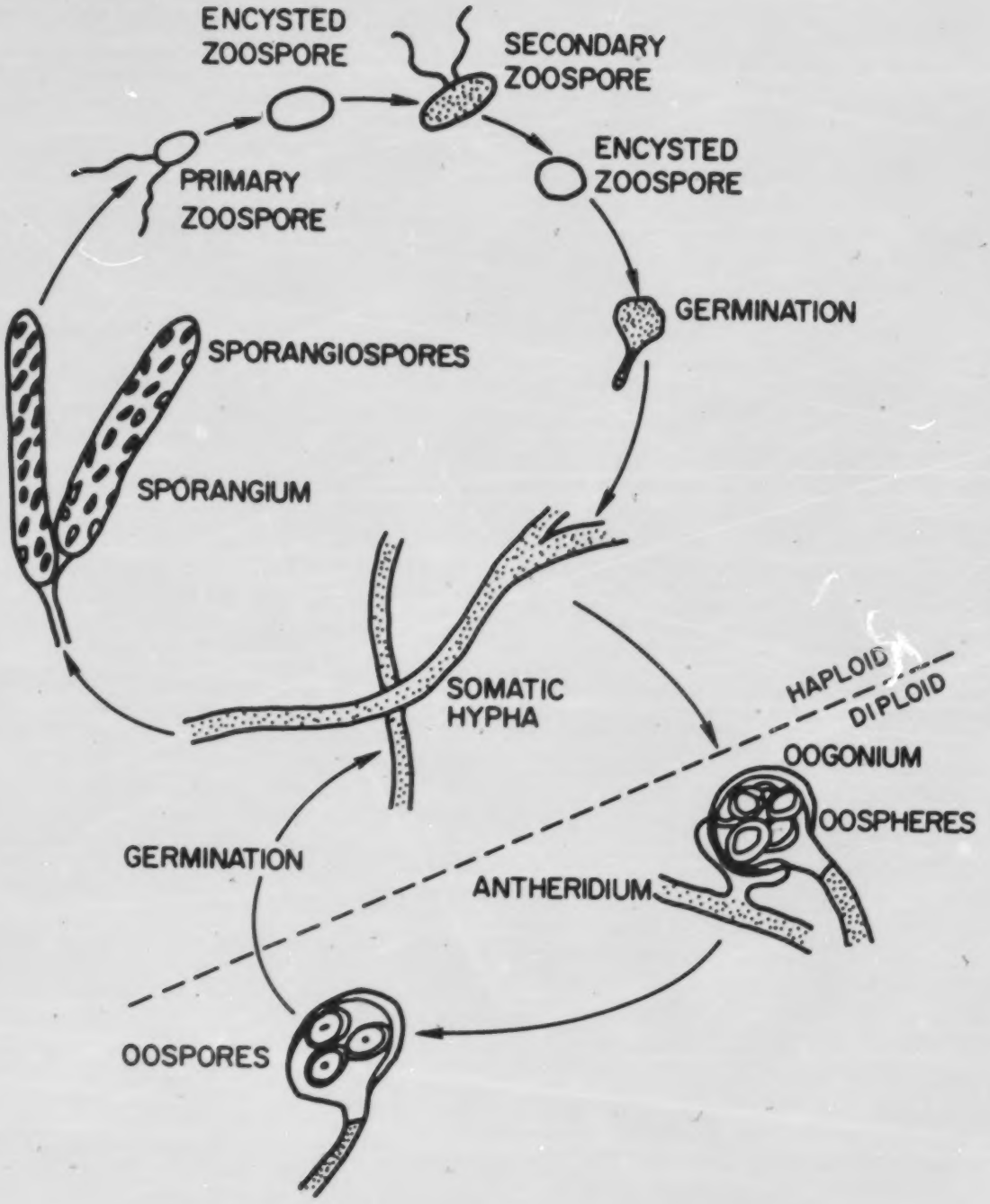


FIGURE I-A-IV. LIFE CYCLE OF *Saprolegnia* SP.

sexual structures oogonium and antheridium (these will be less obvious than the sporangia and are usually found at the end of hyphae). The female organ is a spherical sac-like structure containing the oospheres (eggs). The antheridium (male organ) is a tube that originates from either the same or from a different hypha than the oogonium. This tube penetrates the oogonium, branches out and reaches each of the oospheres. Fertilization takes place through these branched tubes and oospores are formed. The oospores will eventually be released, divide by meiosis and germinate to form new hyphae.

5. Prepare a second wet mount as described above using a few drops of pond water to mount the fungal material. In this way the viable material is observed. Observe the various morphological characteristics of the fungi. In particular, search for motile zoospores inside and outside of their sporangia.

Data Analysis

1. Draw and label the various structures observed in the two preparations of Saprolegnia. Compare your drawings with Figure I-A-IV.

Apparatus

Glass specimen dish with glass cover

Housefly (Musca domestica) freshly-killed by physical means

Glass microscope slides

Cover slips

Dissecting needles

Reagents and Media

Amann's Mounting Medium (Lactophenol Cotton Blue): Dissolve 20 g phenol, 20 g lactic acid and 20 mℓ glycerin in 20 mℓ distilled water and add to this solution Cotton Blue to make the solution 0.1% w/v in Cotton Blue.

EXPERIMENT I-A-4

THE PROTOZOA

Purpose

To introduce the protozoa and develop the ability to recognize the major forms found in aquatic environments.

References

1. Pennak, R.W. Freshwater Invertebrates of the United States. Ronald Press Co., N.Y. 1953.

2. John, T.L. and John, F.F. How to Know the Protozoa, William C. Brown Co., Dubuque, Io., 1949.

Introduction

The protozoa are higher Protista that have traditionally been classified as the lowest forms of the animal kingdom. They have a cellular membrane not associated with a rigid cell wall and a nucleus surrounded in its resting state by a nuclear membrane. More than one chromosome is present in the nucleus; and division and replication of the chromosomes is by mitosis. Plastids, each surrounded by a membrane, are found in the cytoplasm. One type of plastid is the mitochondrion within which aerobic respiratory processes occur. Streaming may be observed in the cytoplasm and motility, if present, is either by amoeboid movement or by use of multistranded, sheathed fibrous organelles called cilia or flagalla.

Most protozoa are single-celled, although some forms consist of a number of cells aggregated into larger units or colonies. The protozoa

show complexity and functional differentiation within the single independent cell. The cytoplasm often contains a number of specialized inclusion bodies such as food vacuoles, oil globules, and contractile vacuoles which serve to regulate the internal osmotic pressure of the cell by water elimination. The protozoan may excrete products such as chitin or cellulose and sometimes a very hard shell or cyst is formed in response to adverse environmental conditions. Portions of the protoplasm may extend out from the cell to form special locomotor appendages. Indeed protozoa are divided into four groups largely based on the type of locomotion.

Superclass Mastigophora of the Subphylum Sarcomastigophora consists of those forms which move by means of one or more long, whip-like extensions called flagella. Many of these are exact couterparts of certain photosynthetic algae and are thought to represent forms transitional in the evolution of the protozoa from an ancestral algal flagellate. Note that although we will classify the photosynthetic flagellates together with other algae, they are often included in the Phylum Protozoa as a distinct class (Phytomastigophora) of Mastigophora.

Superclass Sarcodina (Rhizopods) of the Subphylum Sarcomastigophora consists of those forms which move by means of temporary extensions of the cell called pseudopodia. In effect, the organism moves forward by a flowing of the cytoplasm into these cellular extensions. Amoeba proteus is a typical rhizopod.

Subphylum Sporozoa consists of obligate parasites only. These protozoa have lost their locomotor organelles as a consequence of their parasitic way of life. The class is of great medical importance, and

many are parasitic of fish and other aquatic forms. A well-known example is Plasmodium vivax, one of the malarial parasites.

Subphylum Ciliophora (Ciliates) consists of those forms which move by means of inumerable small hair-like processes called cilia. The cilia are present on all or on part of the cell surface and they beat in a rhythmic fashion. Paramecium caudatum is a typical ciliate. Some ciliates, the Suctorians, show typical locomotion only in an early or immature stage of life. As adults, these organisms lose their cilia and grow long tentacles which are used to suck out the contents of the microbial cells caught as prey.

Protozoa are extremely common in aquatic systems and an especially wide variety can be found where active aerobic decomposition of organic matter is taking place. In small ponds, for example, they may be most easily collected in and around submerged green and rotting vegetation. Protozoa may be saprophytic or holozoic. The former obtain their food by absorption of dissolved organic matter and the latter feed on particulate matter, especially bacteria. Many species are tolerant of low concentrations of dissolved oxygen and, if present can be a useful indicator of the degree and extent of pollution. Only a few protozoa can withstand complete anaerobiosis.

Reproduction in the protozoa may be asexual (binary or transverse fission, multiple fission, or budding). Sexual reproduction may be accomplished in a variety of ways. In many species there is a complete fusion of two haploid cells to form a zygote. A number of amoeba reproduce sexually in this matter. Conjugation (the temporary fusion of two cells with nucleus exchange) is common among the ciliates. In some

instances autogamy (fusion of haploid nucleii from the same cell) occurs.

Protozoa may be readily grown in the laboratory by inoculating boiled infusions of hay and other natural materials with fresh samples of pond water. In mixed cultures different organisms may become predominant in a regular succession as conditions change in the medium. To avoid this, it is often necessary that pure cultures be obtained by techniques involving physical separation and dilution. Bacteria-free cultures are extremely difficult to obtain. Microscopic examination is best performed on fresh, viable material because techniques for preparing permanent mounts frequently distort the specimen.

Examples of various common protoza are shown in Figure I-A-V.

Procedure

1. Collect samples of pond water, preferably from small ponds containing a significant amount of organic matter.
2. Prepare a wet mount slide as describe in Experiment I-A-3 and examine microscopically under low power (10X) for the presence of protozoa. If there are very few specimens present a small volume of pond water may be centrifuged at approximately 100-200 x G and the sedimented material carefully removed and examined microscopically.
3. Fill a finger bowl or specimen dish half full with pond water and add a few grains of rice and allow to stand for a few days. If amoeba are present they will frequently collect about the rice grains. The grains on the bottom of the dish and any associated

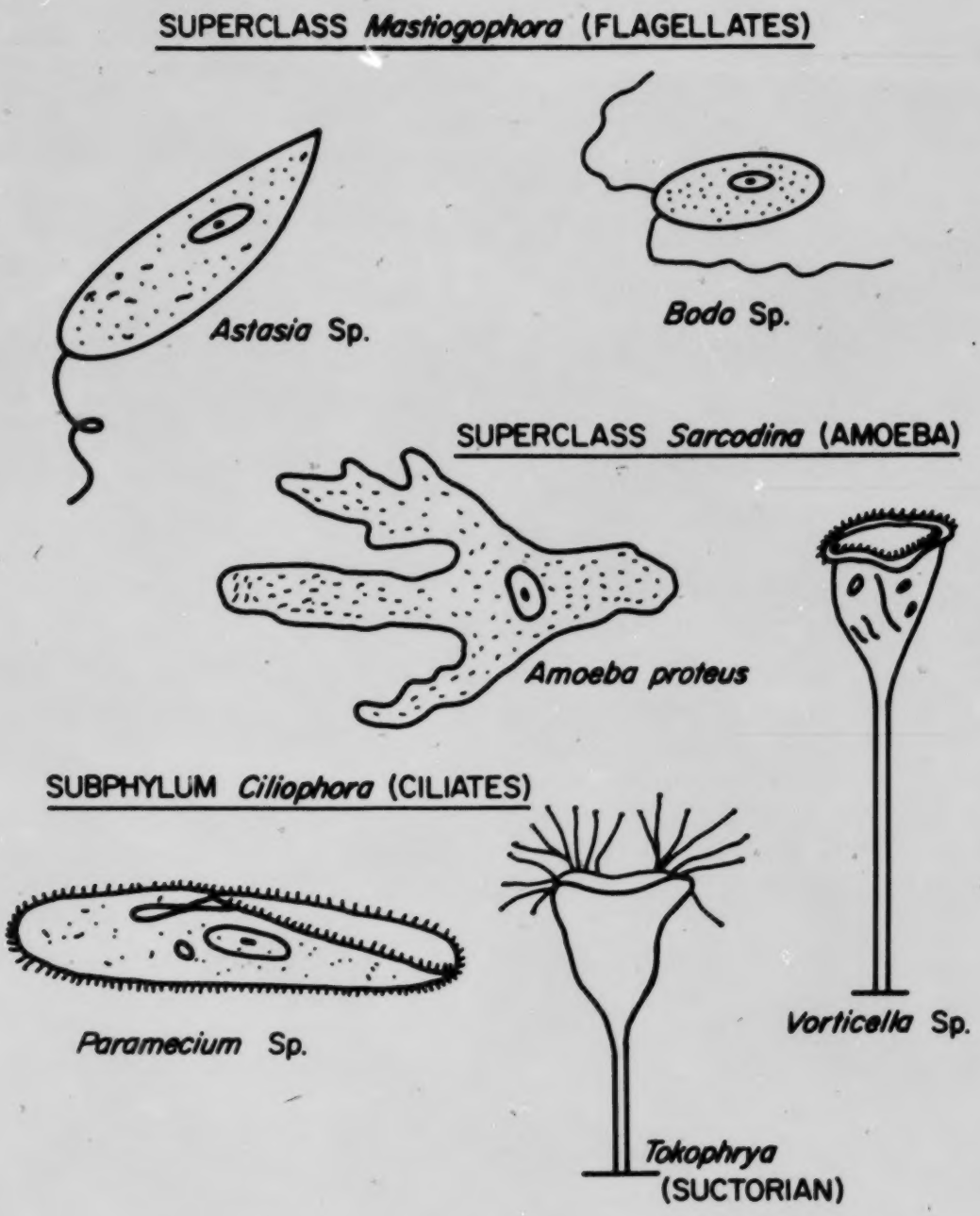


FIGURE I-A-V. EXAMPLES OF COMMON PROTOZOA

amoeba may be observed with a dissecting microscope and the organisms carefully collected with a small pipette.

4. Fast-moving specimens can be better observed by mixing a drop of methylcellulose with a drop of sample or sample concentrate. This procedure increases the viscosity of the suspension so that the movement of the organisms is impeded.

5. Vital staining can be used to increase contrast and facilitate observation of the nucleus and cytoplasmic structures. Evenly distribute the alcoholic Methylene Blue solution on a microscope slide and allow to dry. Place a drop of sample over this dye, mount a cover slip, and observe microscopically. This should stain cytoplasmic granules and the nucleus a blue color. If no staining is obtained it may be necessary to increase the dye concentration.

Data Analysis

Draw and label the various protozoan forms observed. Label the various cytoplasmic inclusions observed. Classify the organisms into their representative categories, i.e. flagellate, amoeba, and ciliate.

Apparatus

Water sampling bottles, 250 mℓ

Centrifuge tubes, 15 mℓ

Glass slides

Cover slips

Finger bowl or shallow dish

Dissecting microscope

Microscope, low power

Pipettes, 1 mℓ

Reagents

Methylcellulose solution: add 10 g methylcellulose (15 centipoise viscosity) to 90 mℓ distilled water.

Methylene Blue staining solution (1:10,000): add 10 mg Methylene Blue to 100 mℓ 95% ethanol.

EXPERIMENT I-A-5

THE ALGAE

Purpose

To observe various species of algae and to become familiar with the differentiation of green algae, blue-green algae and diatoms.

References

1. Standard Methods for the Examination of Water and Wastewater, 14th ed., American Public Health Association, p. 1131, 1976.
2. Prescott, G.W. How to Know the Fresh-Water Algae. William C. Brown Co., Io., 1954.

Introduction

The algae are a mixed group of aquatic and marine life forms that contain chlorophyll. They are important primary producers in the aquatic food web. They are of interest in water treatment where they may cause taste and odor in drinking water supplies and filter clogging in water treatment plants.

Most algae are microscopic, chlorophyll-bearing cells or aggregates of cells although certain aggregates produce extremely large forms such as kelp and other seaweeds. Our interest lies primarily with the microscopic forms.

The term "algae" is broad enough to include members of both the higher and lower Protista. The blue-green algae are procaryotic and are more closely related to bacteria than to fungi or protozoa. They are

without obvious internal cytoplasmic movement; there are no nuclear bodies corresponding structurally to the typical nuclei of the higher Protista and, their photosynthetic pigments are not localized in a discrete body or chloroplast, but are evenly distributed throughout the cytoplasm. In addition to chlorophyll they contain bluish pigments (phycobilins) which together with the green chlorophyll produces a blue-green organism.

The eucaryotic algae, on the other hand, share many cellular characteristics with protozoa and fungi. The major difference is that they are phototrophic (photosynthetic). Their photosynthic apparatus is contained in discrete sub-cellular bodies called chloroplasts.

The algae may be conveniently subdivided into groups as follows:

Chlorophyta. These are green forms that span the diverse range of morphological types from unicellular flagellates to colonial types and from filamentous, to plant-like species. Four cellular pigments (chlorophylls a and b, carotene, and xanthophyll) are present in all species. All species accumulate starch as a reserve material and the cell wall consists of cellulose. Typical species include Chlamydomonas, Chlorella, the colonial form Volvox, and most of the desmids (e.g. Closterium, Desmidium).

Euglenophyta. This is a small group of unicellular organisms with a single anterior flagellum. Instead of a cell wall, the cell is bounded by an elastic pellicle.

Pyrrophyta. Most of these organisms swim using one or two flagella inserted close together on the side of the cell. One of these flagella typically extends backward toward the posterior of the cell while the other lies in a groove that circles the cell. The cell wall is firm and often composed of polygonal plates. The chloroplasts are large, ovoid brown-colored bodies. Most of these organisms are commonly referred to as the armored dinoflagellates. A typical species is Gymnodinium.

Chrysophyta. The most distinguishing characteristic of this group is the cell wall, which is often relatively thick and definite in composition and is often impregnated with silica. Similar to the dinoflagellates, members of this group have brown to yellow-brown chloroplasts. Many unicellular flagellated species exist, but the predominant and most interesting form occurs in the Family Bacillariophyceae, the diatoms. The cell wall of these organisms is a siliceous structure consisting of two overlapping halves that are finely sculptured in unique patterns. Type species includes Navicula and Asterionella.

Rhodophyta. These are exclusively multicellular forms with a characteristic red pigmentation. They are plant-like aggregates that never show flagellation and are often macroscopic.

Phaeophyta. These also are multicellular, large, plant-like forms that are characteristically brown in color and often show a complex

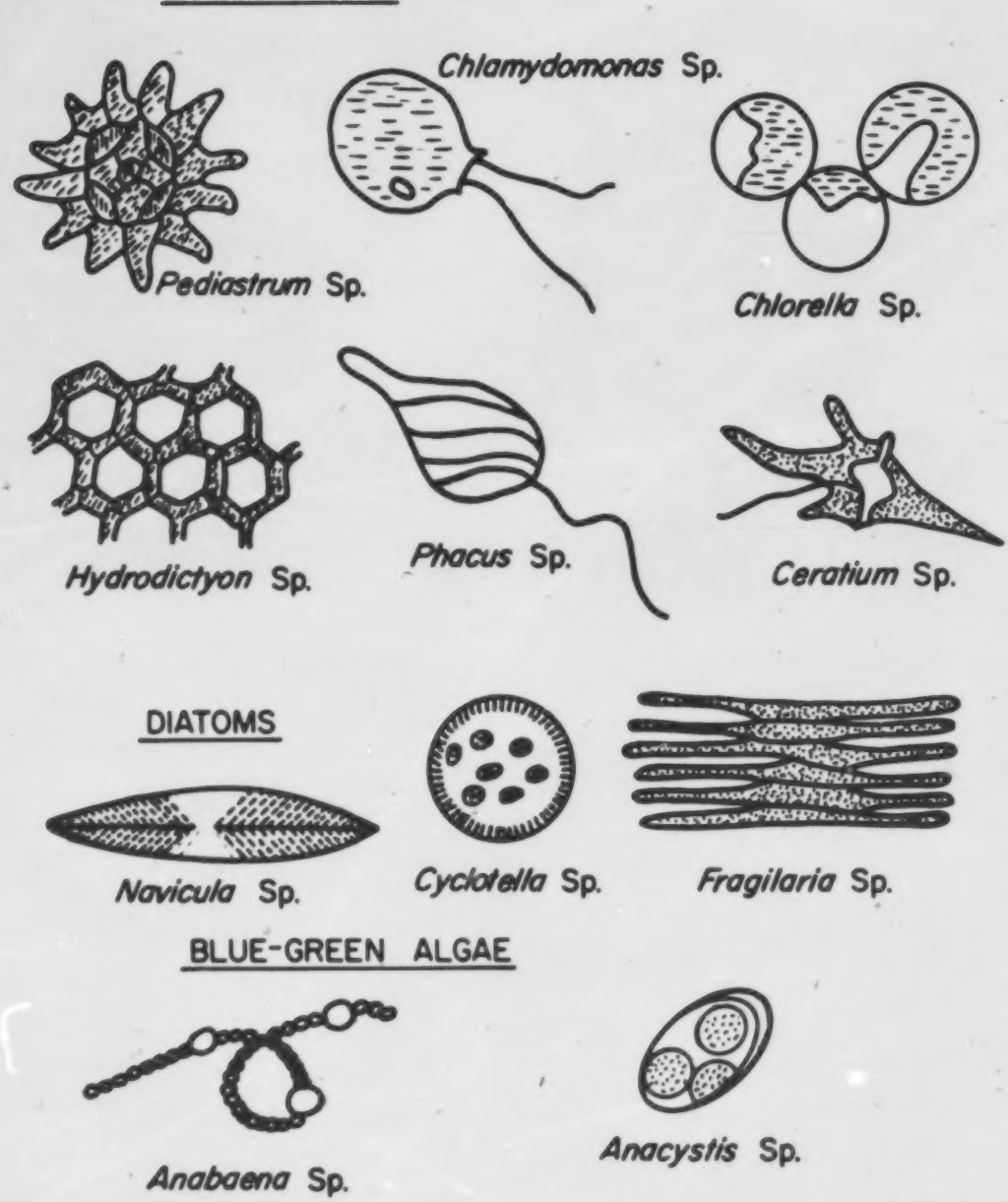


FIGURE I-A- V. EXAMPLES OF COMMON MICROSCOPIC ALGAE

life cycle with alternation of generations and production of flagellated zoospores. The marine kelps are members of this group.

Figure I-A-VI shows sketches of various common algae. The life cycle of the green algae is basically similar to that of the protozoa and the fungi. Some forms reproduce only asexually while others exhibit both sexual and asexual reproduction. The blue-green algae divide only by asexual means.

The algologist differentiates algal species primarily on the basis of morphology. A number of dichotomous identification keys have been developed to assist in species determinations. A good example of such a key is that of Palmer which is used in Standard Methods (Reference 1). Other more complete keys are available.

Procedure

1. Using wet mounts examine algal cultures prepared by your instructor. These may be unialgal cultures obtained from local biological supply houses, material collected from nearby natural waters or from waste stabilization ponds.
2. Identify the types of algae observed. Determine whether they are green, or blue-green algae. This differentiation is based primarily on whether they have or do not have discrete chloroplasts. Color (blue-green vs. green) is helpful but may not be strikingly obvious.
3. Determine if the organisms observed are flagellated, filamentous, or are diatoms. If diatoms are present observe their basic shape, e.g. centric (round) or pennate (cigar shape); observe the delicate

striations on the diatom test (shell); and determine whether or not they are motile.

4. Using a dichotomous algal identification key (Reference 1) identify the algae observed.

Data Analysis

1. Sketch the algal forms studied and indicate on your drawings the essential characteristics used in identification.

Apparatus

Microscope, oil immersion

Glass slides

Cover slips

Materials

Algal cultures

Natural water samples

Waste stabilization pond samples

EXPERIMENT I-B-1

THE EFFECT OF NUTRITIONAL AND ENVIRONMENTAL FACTORS ON GROWTH

Purpose

Growth or reproduction of microorganisms is influenced by a variety of nutritional and environmental factors. Compounds containing carbon, nitrogen and phosphorus are among the essential nutritional requirements. That is, these compounds are required and used for the synthesis of new cellular material. In addition, physical-chemical conditions can influence microbial growth.

The purpose of this experiment is to demonstrate the effect of the following nutritional and environmental factors on the growth of a heterotrophic bacterial population:

1. concentration of organic carbon
2. concentration of nitrogen compounds
3. concentration of phosphorus compounds
4. pH
5. the presence of different hydrogen acceptors

References

1. Stanier, R.Y., Adelberg, B.A. and Ingraham, E. The Microbial World, 4th ed., Prentice-Hall, Inc., Englewood Cliffs, N.J., 1976.
2. Brock, T.D. Biology of Microorganisms, Prentice-Hall, Inc., Englewood Cliffs, N.J., Chapters 4, 5, and 8, 1974.

Introduction

The growth of bacteria can be measured by monitoring either cell counts or cell mass. Bacterial numbers can be measured by plate counting techniques which provide a means of determining viable numbers, while miroscopic observations furnish total numbers of bacteria. Cell mass can be determined by methods such as dry weight, protein analysis, or turbidity measurements. In this exercise, turbidity is used as the index of microbial growth.

The effect of an individual type of nutrient is best seen when all other growth requirements are available in excess. Thus the presence of increasing organic carbon concentrations when nitrogen and phosphorus are in excess will, within limits, elicit an increase in cell growth.

All organisms have an optimal pH region in which they grow best. The pH range of 5-9 is most often found in natural waters. Extremes can sometimes be observed, such as in acid mine water (pH ~ 2) or soda lakes which may have a pH of 11. These conditions, however, are selective for a limited number of species. As this experiment will demonstrate, most organisms prefer the pH range surrounding neutrality.

The oxidation of an organic substrate (the electron donor) can be mediated by bacteria under aerobic conditions with the accompanying reduction of oxygen (the electron acceptor) to water. This is termed aerobic respiration. Under conditions where oxygen is excluded, organic compounds may serve as the electron acceptor. This is commonly referred to as fermentation. Inorganic electron acceptors can be used by some microorganisms in the absence of oxygen (e.g. NO_3^-, SO_4^{-2}) in so-called anaerobic respiration processes. The amount of growth is directly related to the amount of energy released by these different processes.

Procedure

1. The instructor will provide each student group with a series of test tubes labelled as indicated:

   a. A series of tubes containing the following concentration of glucose plus necessary salts (labelled 1-8, respectively): 0, 250, 500, 1000, 2000, 3000, 4000, 5000 mg/ℓ.

   b. A series of tubes containing 5000 mg/ℓ glucose with the following nitrogen to glucose ratios (labelled N1 to N10, respectively): 0, 1:10,000, 1:5000, 1:1000, 1:500, 1:100, 1:50, 1:10, 1:5 and 1:1.

   c. A series of tubes containing 5000 mg/ℓ glucose at the following pH values (labelled P2 to P11, respectively): 2, 3, 4, 5, 6, 7, 8, 9, 10, 11.

   d. A series of tubes containing 5000 mg/ℓ glucose with the following phosphorus to glucose ratios (labelled F1 to F9, respectively): 0, 1:10,000, 1:2000, 1:1000, 1:200, 1:100, 1:10, 1:5, 1:1.

   e. A series of tubes labelled as indicated and containing the following:

      G1. 2000 mg/ℓ glucose,

      G2. 2000 mg/ℓ glucose,

      G3. 2000 mg/ℓ glucose, with nitrates.

      A1. 2000 mg/ℓ acetate acid,

      A2. 2000 mg/ℓ acetic acid,

      A3. 2000 mg/ℓ acetic acid, with nitrates.

2. Pipette 1.0 mℓ of settled sewage into each and every tube. Mix well and replace cotton plugs in all tubes.

3. For tubes G2, G3, A2 and A3, layer melted paraffin wax on top of the liquid media so that air is excluded from contact with the liquid.

4. Incubate all tubes at 35 C temperature for 48 hr.

5. Mix the contents of each tube thoroughly before reading absorbance at 420 nm; use a colorimeter or spectrophotometer. Use tube 1 as the blank for all measurements.

Data Analysis and Questions

1. Present the data in tabular format showing:
   a. the effect of substrate (glucose) concentration on growth
   b. the effect of nitrogen concentration on growth
   c. the effect of pH on growth
   d. the effect of phosphorus on growth
   e. the effect of different hydrogen acceptors on growth
2. Present the data in graphical format showing:
   a. glucose concentration vs. absorbance
   b. N/glucose ratio vs. absorbance
   c. pH vs. absorbance
   d. for each substrate and hydrogen acceptor draw a bar diagram with absorbance as the abscissa. Show bars for each condition of growth with glucose and acetate on the same graph.
3. Did you obtain results which differed from those expected? If so, what factors may have accounted for the difference?
4. Considering the fundamental metabolic pathways involved in the conversion of glucose to CO_2 and water state,

a. why the results of tubes G1 and A1 are similar and why the results of tubes G2 and A2 are dissimilar.

b. what results would you expect if ethanol were the substrate? Explain your answer.

5. Discuss the importance of your observation with respect of anaerobic waste treatment processes.

Apparatus

Colorimeter or spectrophotometer to read at 420 nm.

Test tubes

Erlenmeyer flasks, 250 mℓ, 125 mℓ

Paraffin wax or Vaspar

Phosphate buffer: dissolve 17 g KH_2PO_4, 43.5 g K_2HPO_4 and 66.8 g Na_2HPO_4 in distilled water and dilute to 1 ℓ

Stock Salt Solutions:

1. $MgSO_4 \cdot 7H_2O$ 22.5 g/ℓ
2. $CaCl_2$ 27.5 g/ℓ
3. $FeCl_3 \cdot 6H_2O$ 0.25 g/ℓ

Ammonium Chloride Solutions:

1. NH_4Cl Solution 1; 3.82 g/ℓ
2. NH_4Cl Solution 2; 38.2 g/ℓ

Glucose stock solution: Dissolve 100 g glucose in distilled water and dilute to 1ℓ. Store in a refrigerator.

Tris buffer: Dissolve 24.2 g tris (hydroxymethyl) amino methane in distilled water and dilute to 1ℓ.

HCl; 0.2M.

Media

Refrigerate all media prior to use. Do not autoclave.

1. Glucose media: 1 set per student group:

   Solution 1: Mix together 300 mℓ phosphate buffer solution, 2 mℓ each of stock salt solutions and 1200 mℓ distilled water. Label Solution 1.

   Solution 2: Mix together 300 mℓ NH_4Cl Solution 1 and 1200 mℓ distilled water. Label Solution 2.

Mix equal portions of Solution 1 and Solution 2 and dispense 10 mℓ into each test tube and add glucose stock solution in the following amounts:

Tube No.	1	2	3	4	5	6	7	8
Stock Glucose Solution, mℓ	0	0.25*	0.5*	0.1	0.2	0.3	0.4	0.5

*1:10 dilution of stock glucose solution.

2. Nitrogen media: 1 set per student group

   a. To each test tube add 0.5 mℓ glucose stock solution and 5 mℓ Solution 1 and various concentrations of nitrogen as follows:

Tube		
N1	0	
N2	0.5 mℓ	1:1000 dilution of NH_4Cl Solution 2
N3	1.0	
N4	5.0	

N5	0.5	1:500 dilution of NH_4Cl Solution 2
N6	2.5	
N7	5.0	
N8	0.5	NH_4Cl Solution 2
N9	1.0	
N10	5.0	

b. Bring volumes of all tubes to 10 mℓ with distilled water.

3. pH media, 1 set per student group:

   a. Mix equal portions of Solution 1 and Solution 2.

   b. Adjust pH of combined Solutions 1 and 2 with 50% NaOH or 1:1 H_2SO_4 to yield solutions with pH 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, respectively.

   c. Dispense 10 mℓ into test tubes appropriate labelled P2 - P11.

   d. Dispense 0.5 mℓ glucose stock solution into each test tube.

4. Media for various electron acceptors: 1 set per student group:

   a. Media G1 and G2:

      1) Mix equal portions of Solution 1 and Solution 2.

      2) Add 1.0 mℓ glucose stock solution for every 50 mℓ portion combined Solution 1 and 2.

      3) Dispense 20 mℓ into a 125 mℓ erlenmeyer flask and label G1.

      4) Dispense 10 mℓ into a test tube labelled G2.

   b. Media G3:

      1) To the media prepared in step 4a, add 0.125 g $NaNO_3$ for every 50 mℓ of media.

      2) Dispense 10 mℓ into a test tube labelled G3.

   c. Media A1 and A2:

      1) Mix equal portions of Solution 1 and Solution 2.

2) Add 4.53 g sodium acetate: ($CH_3COONa \cdot 3H_2O$) for every 1ℓ portion of combined Solutions 1 and 2.

3) Dispense 20 mℓ into a 125 mℓ erlenmeyer flask and label A1.

4) Dispense 10 mℓ into a test tube and label A2.

d. Media A3:

1) To the media prepared in step 4c, add 0.125 g $NaNO_3$ for every 50 mℓ media.

2) Dispense 10 mℓ into a test tube and label A2.

5. Phosphorus media: 1 set per student group.

a. Mix 50 mℓ 0.2M Tris buffer and 44.2 mℓ 0.2M HCl; dilute to 100 mℓ. This mixture should have a pH of 7.2.

b. Add 2.0 mℓ of each stock salt solution to each 1500 mℓ portion of Solution 2.

c. Combine equal portions of Tris buffer and the solution prepared in 5b.

d. To each test tube, add 0.5 mℓ glucose stock solution and 5 mℓ combined Tris buffer and the solution prepared in 5c.

e. Make a 1:2.5 dilution of the phosphate buffer, and label "P".

f. Add increasing phosphorus concentrations as follows:

Tube		
F1	0	
F2	1.0 mℓ	1:2000 dilution of "P"
F3	0.5	1:2000 dilution of "P"
F4	1.0	

F5	0.5	1:20 dilution of "P"
F6	1.0	
F7	0.5	Solution "P"
F8	1.0	
F9	5.0	

g. Bring all volume up to 10 mℓ with distilled H_2O.

EXPERIMENT I-B-2

THE MEASUREMENT OF ALGAL GROWTH POTENTIAL USING BATCH CULTURES

Purpose

The purpose of this experiment is to illustrate the stimulating effect of wastewaters on the growth of the blue-green alga, Anabaena flos-aquae. With a bioassay procedure an estimate of the concentration of phosphorus in the wastewater will also be made.

References

1. Provisional Algal Assay Procedures. Joint Industry-Government Task Force on Eutrophication. P.O. Box 3011, Grand Central Station, New York, N.Y. 10017, 1969.
2. Oswald, W.J. and Goankar, S.A. Batch Assays for Determination of Algal Growth Potential. In Proceedings of the Eutrophication-Biostimulation Workshop. Berkeley, California, 1969, pp. 23-38.
3. Standard Methods for the Examination of Water and Wastewater. 14th ed. American Public Health Association. P.744, 1976.

Introduction

Nuisance blooms of algae in receiving waters are often attributable to nutrient enrichment by wastewater effluents. Algal growth assays can provide much information both on the growth-stimulating capacity of a receiving water before and after the input of wastewaters and on the effectiveness of nutrient removal by waste treatment processes. In addition, algal bioassays may permit the determination of the availability of a nutrient that limits algal growth.

In common with other organisms, algal growth or productivity in an aquatic environment is generally considered to be limited by the nutrient which is present in the smallest concentration with respect to the needs of the organism. Because they are photoautotrophic organisms, the growth of algae is primarily limited by availability of the inorganic nutrients, nitrogen or phosphorus. The requirements of different species can vary a great deal. For example, certain species of the Cyanophytes (blue-green algae) have the ability to fix atmospheric nitrogen for their cellular needs. They, consequently, are not growth-limited by very low nitrogen concentrations.

The blue-green alga to be used in this exercise, Anabaena flos-aquae, is a common nuisance organism in impounded waters and is often associated with taste and odor problems in water supplies. Since it is not nitrogen limited, it is a convenient species for determining phosphorus limitations. Maximum algal yields as a function of initial phosphorus concentration will be measured with all other nutrients in excess. The support of algal growth by influent primary settled sewage and secondary effluent (both appropriately diluted), will be assessed.

Procedure

1. Using aseptic techniques, filter all water samples through 0.45 µm membrane filters. The primary settled sewage may require prefiltering through filter paper.
2. Make the following dilutions of both influent and effluent autoclaved wastewater samples with distilled water containing 10 mg/ℓ N in separate flasks: 0, 5, 10, 30, 50, 70% and full strength. Total volume should be 100 mℓ.

3. Add the following concentrations of phophorus reagent to a 50 mℓ portion of Basic Growth Medium (BGM) and bring to a total volume of 100 mℓ: 0, 0.1, 0.2, 0.4, 0.5, 0.6, 0.8, 1.0, 2.0 mg/ℓ.
4. Decant 60 mℓ portions of each wastewater dilution and BGM with added phosphorus to separate 250 mℓ erlenmeyer flasks. Plug with foam or cotton.
5. Centrifuge the Anabaena culture for 10 to 15 min to harvest the cells; wash once with BGM without added phosphorus; resuspend in the same original volume of BGM.
6. Inoculate each flask with 5.0 mℓ of resuspended culture and incubate for 1 week in the light chamber. Shake the flasks two times per day.
7. Harvest algae from each flask by filtering through tared 0.45 µm membrane filters that have been dried overnight at 80 C. Dry the algae overnight at 80 C and weigh to determine algal biomass.

Data Analysis and Questions

1. Using the biomass data, plot the following curves:
   a. Algal dry weight (mg/ℓ) vs. phosphorus concentration (mg/ℓ).
   b. Algal dry weight (mg/ℓ) vs. percent wastewater in the sample. Draw curves for both treated and untreated wastewater on the same graph.
2. Do all the curves originate at zero? If not, how might this be explained?
3. The curves obtained with wastewater can also be interpreted as the Algal Growth Potential (AGP) of that wastewater in mg/ℓ. Discuss the effect of waste treatment on AGP.

4. What is the estimated concentration of phosphorus available for algal growth in both the treated and untreated wastewater samples?

5. What assumptions do you have to make in order to answer Question 4?

Appraratus

Centrifuge tubes, sterile

Pipettes, 10, 1.0, and 0.1 mℓ, sterile

Erlenmeyer flasks, autoclaved, 250 mℓ; 23 per student group.

Light chamber (400 ft-candles)

0.45 μm membrane filters

Aluminum drying cups

Drying oven at 80 C

Acid clean all glassware

Reagents

Nitrate Reagent: Dissolve 7.21 g reagent grade KNO_3 in 1ℓ deionized water. 1 mℓ = 1 mg N

Phosphate Reagent: Dissolve 2.81 g K_2HPO_4 and 2.19 g KH_2PO_4 in 1ℓ deionized water. 1 mℓ = 1 mg P.

Primary settled sewage

Secondary effluent

Basic Growth Medium: Dissolve the following constituents in glass distilled water and autoclave. Prepare 2ℓ per student group.

$MgSO_4 \cdot 7H_2O$	250 mg
$CaCl_2 \cdot 2H_2O$	15.5 mg
$Fe_2(SO_4)_3$	4 mg

$NaHCO_3$	168	mg
Na_2EDTA	50	mg
$MnSO_4 \cdot H_2O$	1.5	µg
$ZnSO_4 \cdot 7H_2O$	200	µg
$CuSO_4 \cdot 5H_2O$	80	µg
H_3BO_3	3	µg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	130	µg
$CoCl_2 \cdot 6H_2O$	40	µg
KNO_3	72.1	mg
Distilled water	1000	mℓ

Anabaena flos-aquae can be obtained at nominal cost from:

a. National Eutrophication Research Program, EPA, 200 SW 35th St., Corvallis, Oregon 97330.

b. Dept. of Botany, University of Texas, Austin, Texas.

The culture should be obtained 2 to 4 weeks in advance, and grown in BGM + 10 mg N/ℓP + 2 mg P/ℓ and transferred 2 to 3 times prior to use.

EXPERIMENT I-C-1

DISSIMILATORY SULFATE REDUCTION

Purpose

The purpose of this experiment is to become familiar with enrichment culture techniques and to observe representative types of bacteria involved in the sulfur cycle. The specific objective of the experiment is to examine the bacteria which reduce sulfates to hydrogen sulfide.

References

1. Skerman, V.B.D. A Guide to the Identification of the Genera of Bacteria, 2nd ed., The Williams and Wilkins Co., Baltimore, 1967.
2. Bergey's Manual of Determinative Bacteriology, 8th ed, Buchanan, R.E. and Gibbons, N.E., eds. Williams and Wilkins Co., Baltimore, 1974.
3. Aaronson, S. Experimental Microbial Ecology, Academic Press, New York, 1970.

Introduction

The transformation of sulfur compounds in nature may be visualized as a cyclic process called the sulfur cycle (Figure I-C-I). The microbes which participate in this cycle are physiologically diverse being both heterotrophic and autotrophic bacteria. Many of the autotrophic bacteria exhibit unique modes of existence and tolerances to environmental conditions.

On a physiological basis, organisms which are capable of reducing sulfate can be classified into two distinct types. Most organisms can reduce sulfate to organic sulfides as demonstrated by their ability to

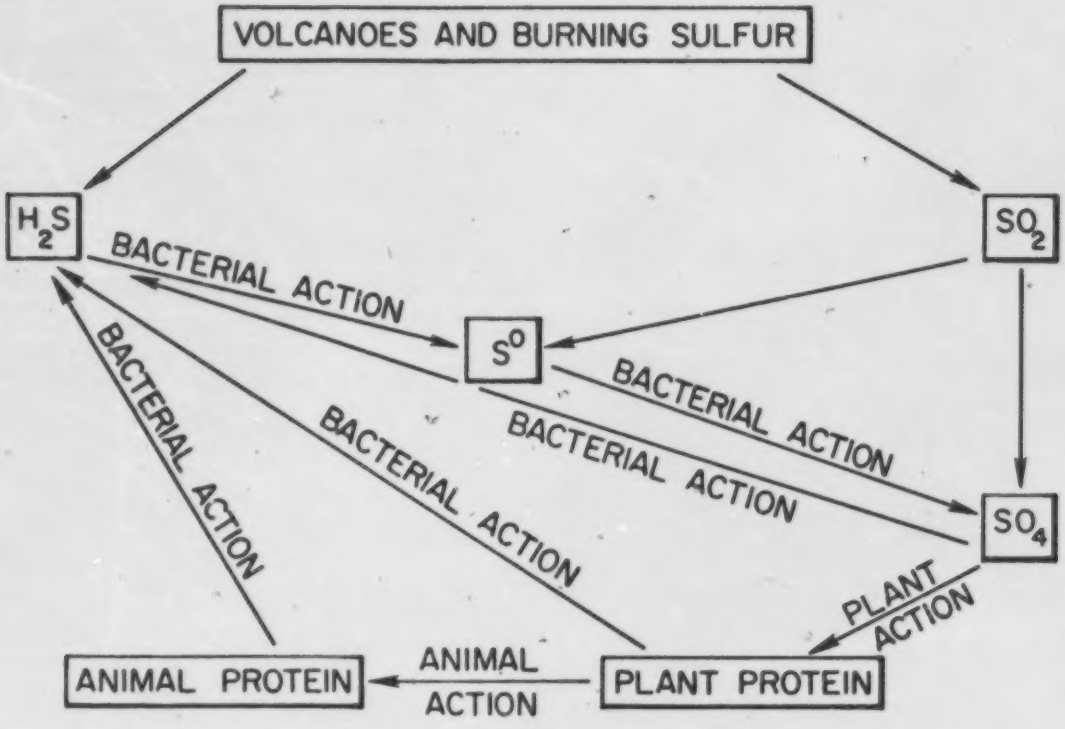


FIGURE I-C-I. THE SULFUR CYCLE

grow using sulfate as a sole source of sulfur. However, they do not form detectable amounts of inorganic sulfide directly from sulfate. This small-scale reduction of sulfate has been termed assimilatory sulfate reduction. Another small group of microbes utilize sulfate as the terminal electron acceptor in anaerobic respiration and produce massive amounts of H_2S. This activity has been termed dissimilatory sulfate reduction.

Organisms which oxidize reduced sulfur compounds can be divided into three groups on the basis of the role which sulfur plays in their physiology: heterotrophic sulfur oxidizers, chemo-autotrophic sulfur oxidizers, and photosynthetic sulfur oxidizers.

The transformation of inorganic sulfur compounds is intimately connected with the energy metabolism of the dissimilatory sulfate reducers and the chemo-autotrophic and photosynthetic sulfur oxidizers.

To detect and isolate these various bacteria (as well as many other environmentally important microorganisms), enrichment techniques are commonly used. These techniques are of inestimable value to the microbiologist and are devised to create environmental conditions that select for those microbes which find such conditions favorable for their proliferation. For example, to detect and isolate bacteria which can reduce sulfates to hydrogen sulfide, one creates conditions in which no oxygen is present, and in which sulfate and an appropriate carbon source are added and incubated. At the end of an appropriate incubation period, a subculture is made into the same medium under the same conditions and the subculture is reincubated. Repeated subcultures are made as necessary. Final isolation is usually accomplished on solid medium of the same composition as the enrichment broths. Figure I-C-II is a schematic diagram of a typical enrichment scheme.

Dissimilatory Sulfate Reducers

The most ubiquitous of these organisms is Desulfovibrio desulfuricans. This organism (and seven others described in the Desulfovibrio species or subspecies) is a strict anaerobe which appears to be obligated to use sulfate as the terminal electron acceptor with the production of hydrogen sulfide. These organisms are heterotrophic and use lower molecular weight organic compounds, such as lactic acid, for their carbon source. The following equation would be representative of their metabolism using lactic acid:

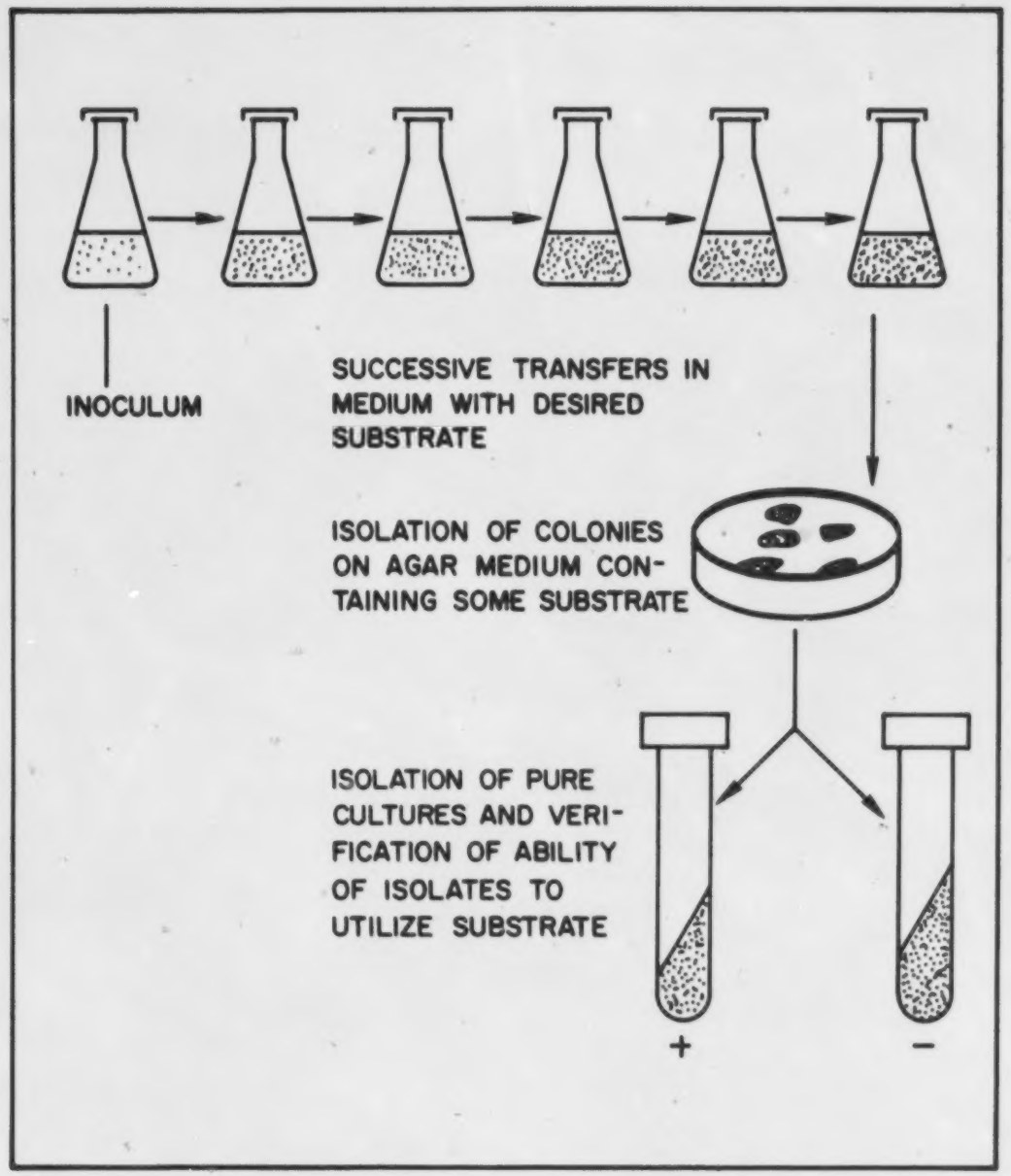

INOCULUM
SUCCESSIVE TRANSFERS IN MEDIUM WITH DESIRED SUBSTRATE
ISOLATION OF COLONIES ON AGAR MEDIUM CONTAINING SOME SUBSTRATE
ISOLATION OF PURE CULTURES AND VERIFICATION OF ABILITY OF ISOLATES TO UTILIZE SUBSTRATE
+
−

FIGURE I-C-II. ENRICHMENT-CULTURE TECHNIQUE FOR ISOLATION OF BACTERIA CAPABLE OF UTILIZING A PARTICULAR SUBSTRATE

$$2CH_3CHOHCOO^- + SO_4^{2-} \rightarrow 2CH_3COO^- + 2HCO_3^{1-} + H_2S$$

lactate → acetate

In nature where anaerobic conditions exist in the presence of sulfates, a great deal of hydrogen sulfide will be produced by these microorganisms. The presence of sulfide may have a significant effect on metals in natural systems by immobilizing them as sparingly soluble metallic sulfides. These organisms may also be involved in the anaerobic corrosion of iron in which the reduction of sulfate acts as the depolarizing step for the oxidation of iron in the absence of oxygen.

There is another genus of bacteria, Desulfotomaculum (three species have been reported), which are gram negative, spore-forming rods which can reduce sulfates in a manner similar to Desulfovibrio. The major member of the group, D. nigrificans, was previously classified as Clostridium nigrificans.

Another source of sulfides which should not be overlooked is from the heterotrophic bacteria which produce hydrogen sulfide during the degradation of organic matter. These organisms are probably the major producers of sulfides from sewage in sewers and sewage treatment plants.

Procedure

1. Enrichment Step

   a. To enrich for Desulfovibrio sp., add approximately 1 g of pond or estuarine sediment to a 300 mℓ BOD bottle.

   b. Completely fill the bottle with Barr's medium to which has been added, just prior to use, 5 mℓ, 1% $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$ per 100 mℓ of medium.

c. Incubate bottle in the dark at room temperature.

d. Observe the bottle during the ensuing laboratory periods, noting the evolution of H_2S by its odor and by the darkening of the liquid medium with metal sulfides. Periodically, remove a small volume of the enrichment culture and prepare a hanging drop-wet mount. Observe the size, shape and motility of the organisms present.

e. An additional or secondary enrichment should be performed by adding a small volume of the enriched material from step 1d into a fresh bottle of Barr's Medium.

2. Isolation of Colonies

a. Make ten-fold serial dilutions of the Desulfovibrio enrichment culture (from Procedure 1 above), using Thioglycollate Broth (Difco) as the diluent. Usually a dilution series of between 1:10 to 1:10,000 is sufficient.

b. Inoculate 0.1 mℓ of each dilution into tubes containing between 9-10 mℓ of melted Starkey's Agar (held at 46 C) to which 1 mℓ of a sterile solution of 0.1% w/v of thioglycollic acid has been added. Mix carefully and allow to solidify.

c. Incubate in the dark at room temperature until black colonies appear suspended in the agar.

d. The agar may be extruded from the tube by running a small diameter tube (Pasteur pipette) between the glass wall and the agar. Apply a small amount of air pressure on the tube and force the agar out into a culture dish.

e. Carefully pick a colony and examine the colonial material in a wet mount (hanging drop).

Data Analysis

1. Record the results of the enrichment culture procedure. Describe the presence or absence of hydrogen sulfide and describe the types of bacteria seen during the microscopic examination. Make note of the relative numbers of motile, comma-shaped bacteria present. You may see a large number of what appears to be longer spiral-shaped forms. These usually are Desulfovibrio which have divided but not separated. Make a record of the colonial growth of the bacteria isolated from the enrichment step. Compare these "pure" cultures to the microorganisms seen in the enrichment culture. The presence of non-sulfide producers if any should be noted.

Apparatus

BOD bottles, 300 mℓ

Hanging drop slides

Cover slips

Pasteur pipettes

Reagents

1. Barr's Medium

K_2HPO_4	0.5 g
NH_4Cl	1.0 g
$CaSO_4$	1.0 g
$MgSO_4 \cdot 7H_2O$	2.0 g
NaCl	10.0 g
70% w/v sodium lactate solution	5.0 g
Tap water	1000 mℓ

Dissolve ingredients in 1000 mℓ of water, adjust pH to 7.5, and autoclave at 121 C for 15 min.

Separately prepare a 1% w/v solution of $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$ and filter sterilize. Aseptically add 5 mℓ of this solution to each 100 mℓ of medium, just prior to inoculation. This medium may form a precipitate but it will still be suitable for enrichment.

2. Starkey's Agar Medium

K_2HPO_4	0.5 g
NH_4Cl	1.0 g
Na_2SO_4	1.0 g
$CaCl_2 \cdot 2H_2O$	0.1 g
$MgSO_4 \cdot 7H_2O$	2.0 g
70% w/v sodium lactate solution	5.0 g
Distilled water	1000 mℓ

Dissolve the constituents in 1000 mℓ distilled water, boil this solution for 10 min and filter out any precipitate that forms. Add 20 g of agar per 1000 mℓ of medium. Completely dissolve and autoclave at 121 C for 15 min. Cool to between 45-50 C and aseptically add 5 mℓ of a 1% solution of $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$ per 100 mℓ of agar medium. Aseptically dispense 9 mℓ of this medium to culture tubes and hold at 45 C for inoculation. Prior to inoculation, add 1 mℓ of 0.1% w/v of filter sterilized thioglycollic acid solution.

EXPERIMENT I-C-2

OXIDATION OF REDUCED SULFUR COMPOUNDS

Purpose

The purpose of this experiment is to enrich for the important bacteria involved in the oxidation of reduced inorganic sulfur compounds, to observe their chemical activity and to examine their morphology. This experiment will also serve to introduce chemoautotrophic and photoautotrophic bacteria.

References

1. Bergey's Manual of Determinative Bacteriology, 8th ed. Buchanan, R.E. and Gibbons, N.E., eds., Williams and Wilkins Co., Baltimore, 1974.
2. Skerman, V.B.D. A Guide to the Identification of the Genera of Bacteria, 2nd ed., Williams and Wilkins Co., Baltimore, 1968.
3. Rodina, A.G. Methods in Aquatic Microbiology. University Park Press, Baltimore, 1972.
4. Zajic, J.E. Microbial Biogeochemistry, Academic Press, New York, 1969.
5. Standard Methods for the Examination of Water and Wastewater, 14th ed., American Public Health Association, p. 319, 1976.

Introduction

The formation of elemental sulfur and various other higher oxidation states of sulfur by the microbial oxidation of more reduced forms of

sulfur is a common activity in nature. In certain situations such activity can result in unsightly growths of filamentous bacteria or acid conditions that adversely affect other forms of life. These activities may also mobilize metals by oxidizing or solubilizing their metallic sulfides. There are a variety of types of microorganisms capable of such activities including chemoautotrophs, chemoheterotrophs and photoautotrophs.

The most studied chemoautotrophs involved are members of the genus Thiobacillus. Their general chemical activity can be described by the following:

$$2S^\circ + 3O_2 + 2H_2O \rightarrow SO_4^{2-} + 4H^+$$

Sulfur Sulfate

$$5S_2O_3^{2-} + H_2O + 4O_2 \rightarrow 6SO_4^{2-} + 4S^\circ$$

Thiosulfate Sulfate Sulfur

They are all aerobes, their energy is gained from the oxidation of sulfur compounds, their carbon source is CO_2. One species, T. denitrificans, will grow anaerobically in the presence of nitrate, using the latter as the electron acceptor rather than oxygen as shown in the following equation:

$$6NO_3^{1-} + 5S^\circ + 2H_2O \rightarrow 5SO_4^{2-} + 3N_2 + 4H^+$$

Nitrate Sulfur Sulfate Nitrogen Gas

It is evident from these equations that acid conditions will be produced by the metabolic activities of these microbes. It has been estimated that T. thiooxidans must oxidize 31 grams of sulfur for each gram of carbon assimilated. The tolerance of these bacteria to low pH varies with species. Thus, T. thiooxidans has an optimum growth pH between 2

and 3 and may grow at pH values of less than one. Thiobacillus thioparus, has a pH optimum near 7 but can grow at pH 5. One species, T. ferroxidans, can also gain energy through the oxidation of iron salts.

A number of filamentous bacteria can oxidize sulfides to elemental sulfur and sulfate. The most well-known is Beggiatoa alba which commonly grows in polluted water and other situations enriched with hydrogen sulfide. This aerobic microorganism gains energy for growth through the oxidation of sulfide but needs an organic carbon source. Microscopically, it resembles a filamentous blue-green alga containing inclusions of elemental sulfur but does not produce chlorophyll. It demonstrates a creeping motility. Thioploca sp. have a similar metabolic pattern to Beggiatoa sp. but rather than being single filaments they appear as bundles surrounded by a sheath. A number of less commonly recognized filamentous forms including Thiothrix sp. oxidize H_2S. They typically grow in rosettes and are considered to be chemoautotrophic.

The student should be aware that there are at least twelve genera of bacteria which contain species that oxidize sulfur aerobically. For more details, see Reference 1.

Reduced inorganic sulfur can be oxidized under anaerobic conditions through the metabolic activity of purple and green sulfur photosynthetic bacteria. In this instance light is the energy source and hydrogen sulfide acts as the reductant for the transformation of carbon dioxide to cellular material. This reaction can be generalized as follows:

$$2H_2S + CO_2 \xrightarrow{\text{light}} CH_2O + 2S^\circ + H_2O$$

Sulfide (cells) Sulfur

The purple sulfur bacteria (Chromatiaceae) contain bacterial chlorophyll, carotenoids and absorb light in the infra-red range between 800-900 nm. They will not grow in light which does not contain sufficient infra-red such as a fluorescent lamp. The sulfur produced is usually stored inside the cells. Thus, when a healthy culture is microscopically observed, large sulfur inclusions are an obvious characteristic. In one genus the sulfur granules are outside the cell. Reference 1 lists 10 genera with 26 species of purple sulfur bacteria.

The classification of these forms into genera is based primarily on the characteristics of single cells (e.g. coccus, rod, spiral), arrangement of cell masses, the presence or absence of gas vacuoles and whether elemental sulfur is found inside or outside the cell. The type genus is Chromatium which consists of single, motile cells.

Massive growths of these cells may be found in certain sulfurous lakes, hot springs, and in anaerobic lagoons in which concentrated organic wastes are being treated.

The green sulfur bacteria (Chlorobiaceae) have a metabolism similar to the purple sulfur bacteria, contain a bacteriochlorophyll, and specific carotenoids. Sulfur is always deposited outside the cell. Five genera have been described. These bacteria occur in similar situations to the purple sulfur bacteria, but because of their color, their presence is less dramatic than the presence of purple bacteria.

Procedure

1. Enrichment for Thiobacillus

   a. Prepare four 250 mℓ erlenmeyer flasks, two of which each

contain 100 mℓ of Parker and Fisk Medium I (PFI) and two of which each contain 100 mℓ of Parker and Fisk Medium II (PFII).

b. Inoculate one flask of each medium with 5 g pond or similar sediment and keep the second flask of each medium as a control.

c. Incubate all flasks in the dark at room temperature. The use of a shaker is helpful although not necessary.

d. Initially and at two or three day intervals over a two-week period remove 5 mℓ from each flask and titrate with a standard 0.1 N iodine solution to determine thiosulfate. As the incubation continues, the titration may better be performed with an 0.01 N iodine solution. Measure the pH of each sample prior to thiosulfate determination.

e. When good growth has occurred as evidenced by the accumulation of elemental sulfur, by the turbidity produced by increases in bacterial numbers and by the removal of at least 50% of the thiosulfate, transfer 1 mℓ of the culture to a flask of the same medium and reincubate. This is the second enrichment.

f. Determine thiosulfate removal and pH changes on this second enrichment in the same manner as in Step 1d above. After good growth has occurred, streak PF I and PF II agar plates with material from the enrichment culture. Incubate the plates at room temperature and observe them periodically for colony development. Prepare a hanging drop slide using material from the colonies formed on PF I and PF II agar and examine for motility. Also prepare a gram stain of this material. Before discarding the agar plates, flood them with a pH indicator

such as Brom Cresol Purple and note the production of acid surrounding the colonies.

2. Enrichment for Photosynthetic Sulfur Bacteria

   a. Place enough pond sediment or similar materials into two 300 mℓ BOD bottles so that about a 3-cm layer is formed on the bottom of the bottles.

   b. Fill the BOD bottles completely with Van Niel's medium. Insert the glass stoppers to exclude air and incubate at room temperature. Illuminate the bottles with incandescent light.

   c. During a period of about 3-4 weeks, observe the bottles for the growth of green and purple sulfur bacteria. These may occur just on the surface of the sediment or throughout the entire bottle.

   d. When growth has occurred, observe the material microscopically using a hanging drop preparation.

Data Analysis

1. Record your observations of the enrichment of Thiobacillus sp. Tabulate the change in concentration of thiosulfate with incubation time. Also record pH changes. Present these data graphically; note the differences in rates of change between the first and second enrichments. Note any differences seen in the growth on PF I and PF II medium. The former should be selective for more aciduric strains. Describe the types of bacteria isolated on the PF I and PF II agar media.

2. Describe the results of the enrichment for photosynthetic sulfur bacteria. In particular, note the color of masses of these microbes,

the presence or absence of sulfur inclusions, and the morphology of the individuals involved.

Apparatus

Erlenmeyer flasks, 250 mℓ

Well slides

Petri dishes

BOD bottles, 300 mℓ

Incandescent light source, 60 watt bulb.

Reagents

Standard Iodine Solution: 0.1 N; dissolve 40 g KI in 25 mℓ distilled water, add 13 g resublimed I_2; stir to dissolve; dilute to 1ℓ with distilled water, standardize against sodium arsenite (Reference 5, page 319).

Standard Iodine Solution: 0.01N; dissolve 16 g KI in distilled water and add 100 mℓ standard 0.1 N iodine solution; dilute to 1ℓ with distilled water; prepare fresh; store in dark.

Starch Indicator: 0.5% soluble starch in distilled water. Heat to dissolve.

1. Parker and Fisk Medium I (PF I)

$Na_2S_2O_3 \cdot 5H_2O$	5 g
$CaCl_2$	0.1 g
KH_2PO_4	3.0 g
$MgCl_2 \cdot 6H_2O$	0.1 g
NH_4Cl	0.1 g
Distilled water	1000 mℓ

Dissolve all ingredients except the sodium thiosulfate in distilled water and sterilize by filtration or autoclaving. If sterilization is by autoclaving filter sterilize the sodium thiosulfate and add aseptically to autoclaved material.

2. Parker and Fisk Medium II (PF II)

$Na_2S_2O_3 \cdot 5H_2O$	10.0 g
$MnSO_4 \cdot 4H_2O$	0.02 g
$FeCl_3 \cdot 6H_2O$	0.02 g
$CaCl_2$	0.1 g
$MgSO_4 \cdot 7H_2O$	0.1 g
KH_2PO_4	4.0 g
K_2HPO_4	4.0 g
$(NH_4)_2SO_4$	0.1 g
Distilled water	1000 mℓ

Sterilize as for PF I medium.

3. PF I and II Agar

Prepare solutions as for PF I and PF II media; add 15 g agar to the ingredients. As for PF I and PF II media filter sterilize the sodium thiosulfate in a concentrated solution and add aseptically to the melted agar mix.

4. Van Niel's Medium

NH_4Cl	1.0 g
K_2HPO_4	0.5 g
$MgCl_2$	0.2 g
$NaHCO_3$	0.5 g
$Na_2S \cdot 9H_2O$	1.0 g
NaCl	15.0 g

$CaCl_2$	0.05 g
$FeCl_3 \cdot 6H_2O$	0.05 g
Tap water	1000 mℓ

This medium is not sterilized.

EXPERIMENT I-C-3

THE WINOGRADSKY COLUMN

Purpose

To simulate and intensify certain natural conditions in which the enrichment and interaction of mixed bacterial species may be observed.

References

1. Winogradsky, S. "Recherches, Physiologiques sur les Sulfobacteries." Annales de l'Institut Pasteur, 3:49-60, 1889.
2. Brock, T.D. Principles of Microbial Ecology, Prentice-Hall, Inc. New Jersey, 1966.

Introduction

The study of microbial ecology in a laboratory can be rather complicated, so one usually works with pure cultures of microorganisms and notes the chemical conversions carried out by an individual species. However, the chemical changes observed in nature are the end result of chains of microbial interactions. The purpose of this exercise is to attempt to duplicate some of the natural conditions in which conversions of carbon and sulfur compounds may be observed.

The classic study of Winogradsky in 1889 deserves repetition today because it is one of the simplest methods for demonstrating the ecology of a number of different microorganisms.

A modified version of this study may be achieved by filling a glass cylinder about 3 in. deep with a mixture of estuarine or pond mud,

calcium sulfate and shredded filter paper, and covering this mixture with several volumes of water.

The cylinder is then incubated at room temperature in either natural or incandescent light.

The following interactions are hypothesized:

a. Desulfovibrio sp. grows anaerobically in the mud bottom and reduces sulfate to hydrogen sulfide. Organic compounds are oxidized.

b. Fermentative organisms recycle the organic compounds by oxidizing some to CO_2 and reducing others.

c. Anaerobic photosynthetic purple and green sulfur bacteria grow in the reduced mud and oxidize the sulfides to sulfur and sulfate. They use CO_2 as their carbon source (some may also utilize organic carbon). Since light serves as the source of energy, these organisms frequently grow in the interstices between mud and glass.

d. Photosynthetic non-sulfur bacteria are more tolerant of oxygen and hence may be found nearer the surface of the cylinder. They obtain energy from the light and use organic carbon.

e. Green algae may also develop in the liquid area. Using energy from light, they convert CO_2 to organic material and produce oxygen.

f. In the upper region of the liquid, aerobic chemoorganotrophs may grow using oxygen as their hydrogen acceptor and oxidizing organic compounds.

In this region one may also observe the growth of filamentous, colorless sulfur bacteria such as Beggiatoa sp.

Procedure

1. Mix approximately 250 mℓ of mud with approximately 25 g of hay or shredded filter paper and 5 g $CaSO_4$.
2. Place the mixture in a 1000 mℓ cylinder. Fill with water from the same area from which the mud was taken and allow the mixture to settle. (Use marine water preferentially, if available).
3. Label the cylinder and incubate at room temperature in the light. Growth will become evident in a few days.
4. Observe the column during each lab period for a period of up to 8 weeks and record your observations.
5. Make macroscopic and microscopic observations at the following regions:
   a. At the upper surface of the mud sediment look for purple and green layers of growth. These are the photosynthetic purple and green sulfur bacteria.
   b. Within the upper middle portion or surface of the column look for filamentous, colorless sulfur bacteria.
   c. At the surface of the water column look for purple non-sulfur bacteria.
   d. Note the production of sulfides either by the presence of black metal sulfides or by the odor of hydrogen sulfide.
   e. Using long glass tubes remove small samples of the various growths and examine microscopically.

Results

Carefully record your observations, noting time and location of appearance of various types of microbial life. Describe, using drawings,

the cellular morphology of the microbial types which develop. Briefly describe the metabolic processes involved for each type of microorganism observed in the column including chemical equations which illustrate oxidation and reduction reactions.

Apparatus

Glass cylinders, 1ℓ

Filter paper or hay

Glass tubing

Estuarine or pond mud

Estuarine or pond water

EXPERIMENT I-D-1

NITROGEN FIATION

Purpose

The purpose of this experiment is to measure the activities of nitrogen fixing microorganisms. The growth and morphological response of these organisms in an environment free of combined nitrogen will be observed.

References

1. Rodina, A.G. Methods in Aquatic Microbiology, Univ. Park Press, Baltimore, 1972.
2. Alexander, M. Microbial Ecology, John Wiley and Sons, N.Y., 1971.

Introduction

Denitrification is the major pathway in the nitrogen cycle by which nitrogen is returned to the atmosphere from the hydrosphere. Counter to this phenomenon is the process of nitrogen fixation by which gaseous nitrogen is reduced by microorganisms to nitrogen containing cellular material. It has been estimated that about 10^8 tons/yr of nitrogen is fixed over the surface of the earth and that approximately 2×10^7 tons of nitrogen is returned to the atmosphere annually from marine denitrification alone.

There are not many known species of microorganisms which can fix nitrogen and no eucaryotic organisms with this capability have been found. There are a few bacterial species and a number of blue-green

algae which can fix nitrogen. Among the bacteria there are two distinct groups - those which grow independently (for example, Azotobacter, Clostridium pasteurianum) and those which fix nitrogen only in symbiotic relationship with leguminous plants. This latter group are members of the genus Rhizobium and although of great importance in agriculture are not common to the aquatic environment. Azotobacter sp., a heterotrophic aerobe, and Cl. pasteurianum, an anaerobic heterotroph, are commonly associated with water and sediments. These organisms are not obligated to nitrogen fixation and in the presence of a combined nitrogen source, such as ammonia, will grow without the fixation of nitrogen.

The biochemical pathway of nitrogen fixation is not well understood at the present time. It would appear, however, that the nitrogen is reduced (in the cell) to ammonia and then incorporated into amino acids and proteins. This process requires a significant expenditure of energy since six electrons are required to reduce N_2 to $2NH_3$.

A number of blue-green algae fix nitrogen. All are heterocystous and most algologists agree that the heterocyst is the site of nitrogen fixation. As with the nitrogen fixing bacteria, the algae are not obligated to this mode of nutrition but will readily use ammonia and nitrate when present. In this experiment the heterocystogenic response of the blue-green algal genus Anabaena (will be observed) in media with and without combined nitrogen present.

Procedure

1. Nitrogen Fixation by Bacteria

   An enrichment technique for the growth of nitrogen fixing bacteria will be employed. In one instance a known culture of Azotobacter

vinlandii ATCC 13518 will be used. In the second instance soil or sediment will be used as the source of nitrogen fixing bacteria.

a. Inoculate 250 mℓ flasks containing 50 mℓ of sterile nitrogen-free medium (NFM) with 1 mℓ of a culture of Azotobacter vinlandii. This culture should have previously been grown and transferred a number of times in the same nitrogen-free mineral medium. Incubate one flask at room temperature (or 25 C) on a rotary shaker.

b. Inoculate a second 250 mℓ flask containing 50 mℓ of NFM with 1 g soil or pond sediment. Incubate on a shaker at room temperature (or 25 C) for 5 days. At the end of this time transfer 1 mℓ of this culture to a fresh flask of medium and reincubate. At the end of 3 to 5 days, again transfer 1 mℓ to fresh medium and incubate. To save time the instructor may carry out the first two enrichments and the student will then be responsible for only the third subculturing.

c. At the time of inoculation (zero time) take a one mℓ portion of either the pure culture (A. vinlandii) or of the third enrichment inoculum, dilute with 20-30 mℓ NFM culture medium and filter through a preweighed, 0.45μm, 47 mm diameter membrane filter. Dry the filter at 80 C for 12 hr (overnight), cool, weigh and determine the biomass present.

d. At the end of 5 days incubation, determine the biomass present as in Step 1c. Prior to the biomass determination, spread a loop full of each culture on a microscope slide, air dry, heat fix, stain with Gram Crystal Violet for 1 min, wash with tap water, blot dry, and examine under the microscope.

2. Nitrogen-Fixation by Algae

a. Anabaena sp. is a well-known nitrogen fixing blue-green alga. In this procedure rather than measuring biomass increase in a nitrogen-free media, morphological changes which are related to nitrogen-fixation, i.e., heterocyst formation, will be examined.

b. To a 250 mℓ erlenmeyer flask containing 100 mℓ of Anabaena Medium (AM), add 1 to 2 mℓ of an actively growing culture of Anabaena sp. that has been prepared in AM medium by the instructor. Incubate at room temperature with illumination using a rotary shaker if available.

c. To a second 250 mℓ flask, add 100 mℓ of modified nitrogen-free AM medium. Inoculate as described in Step 1b.

d. After sufficient growth has occurred, microscopically examine small samples from each flask using a wet mount method. Examine a number of algal chains and determine the proportion of heterocysts to normal cells for the Anabaena grown in each flask.

Results

1. Record the changes in bacterial biomass produced in the absence of any added nitrogen compounds in the growth medium. From the amount of biomass produced, estimate the amount of nitrogen fixed. Compare your microscopic examinations of the bacterial growth in each of the flasks. Did you enrich a single or multiple type of bacteria from the soil or sediment sample? Discuss what critical factors

would be involved in determining the type of microorganism selected for.

2. Note the relationship between the number of heterocysts and the number of "normal" cells produced when the blue-green alga, Anabaena, is grown with and without an added nitrogen source. Was there any obvious difference in the biomass of algae produced?

Apparatus

Compound microscope

Well slide

Cover slips

Shaker table

Membrane filters, 0.45 µm, 47mm diameter

Membrane filtration apparatus

Illumination source, 400 ft-candles.

Reagents

1. Gram Stain, See Experiment I-A-2
2. Nitrogen-free medium (NFM)

Mannitol	15 g
K_2HPO_4	0.5 g
$MnSO_4$	10 mg
$FeCl_3$	10 mg
$CaCO_3$	5.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
NaCl	0.2 g
Distilled water,	1000 ml

Filter Sterilize

3. Anabaena medium (AM)

$MgSO_4 \cdot 7H_2O$	250 mg
$CaCl_2 \cdot 2H_2O$	15.5 mg
$Fe_2(SO_4)_3$	4 mg
$NaHCO_3$	168 mg
Na_2EDTA	50 mg
$MnSO_4 \cdot H_2O$	1.5 mg
$ZnSO_4 \cdot 7H_2O$	200 mg
$CuSO_4 \cdot 5H_2O$	80 mg
H_3BO_3	3 mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	130 mg
$COCl_2 \cdot 6H_2O$	40 mg
K_2HPO_4	5 mg
KH_2PO_4	5 mg
Distilled water	1000 mℓ

Autoclave or filter sterilize

4. Modified Anabaena medium (MAM)

Same as AM with the addition of 37 mg of KNO_3 per 1000 mℓ of medium.

5. Anabaena spp (e.g. Anabaena flos-aquae) can be obtained at nominal cost from:

a. National Eutrophication Research Program, EPA, 200 S.W. 35th Street, Corvallis, Oregon 97330.

b. Dept. of Botany, University of Texas, Austin, Texas.

EXPERIMENT I-D-2

AMMONIFICATION AND NITRIFICATION

Purpose

The purpose of this experiment is to observe and measure the production of ammonia from an organic compound (ammonification) and the subsequent bacterial oxidation of ammonia to nitrite and nitrate (nitrification).

Reference

1. Standard Methods for the Examination of Water and Wastewater, 14th ed., American Public Health Association, pp.412, 429 and 434, 1976.

Introduction

The nitrogen cycle (Figure I-D-II) is of global importance and of considerable interest to those involved in water quality management. Many bacteria and algae are involved in the cycling of nitrogen.

Ammonification is the production of ammonia by microorganisms and higher life forms as the result of the degradation of nitrogen containing organic material. This process may occur under both aerobic and anaerobic conditions. Many common heterotrophic bacteria degrade nitrogen containing organic matter with the production of ammonia, particularly when there is more nitrogen present than required for the organism's biosynthesis.

Nitrification is the oxidation of ammonia to nitrite then from nitrite to nitrate and is primarily carried out by a number of genera of

autotrophic bacteria: Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosocystis, Nitrosogloea, Nitrobacter, and Nitrocystis. The first five genera gain their energy from the oxidation of ammonia to nitrite while the latter two oxidize nitrite to nitrate thus:

$$2\ NH_3 \xrightarrow{+O_2} 2NH_2OH \xrightarrow{-4H} HON = NOH \xrightarrow{+O_2} 2\ HNO_2 \quad (\Delta Kcal = 79)$$

$$HNO_2 \xrightarrow{+H_2O} HON\begin{matrix}OH\\OH\end{matrix} \xrightarrow{-2H} HNO_3 \quad (\Delta\ Kcal = 216)$$

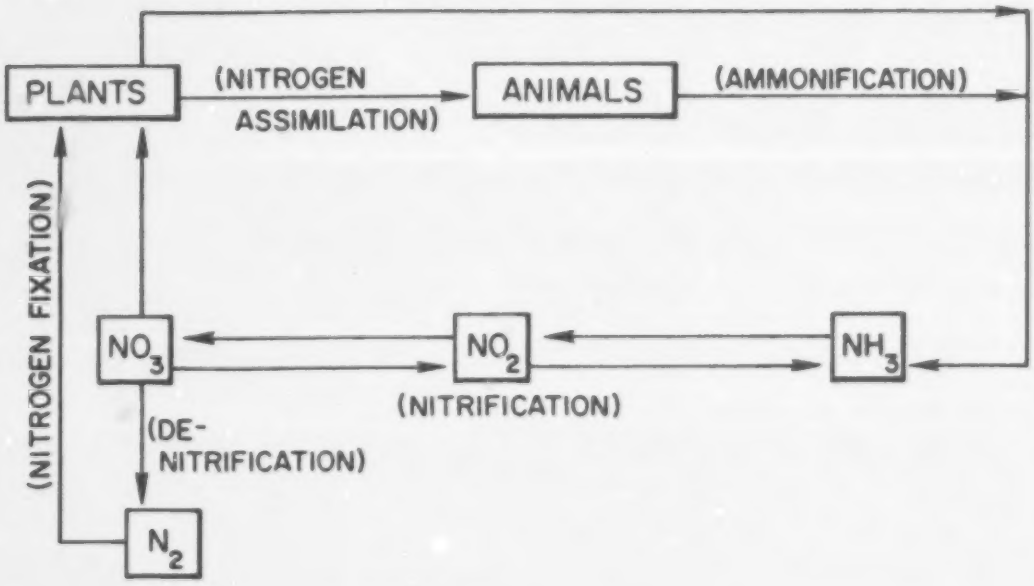


FIGURE I-D-II. THE NITROGEN CYCLE

Both groups use carbon dioxide as a carbon source. The ratio of nitrogen oxidized to carbon reduced is 35:1 for the ammonia oxidizers and as high as 135:1 for the nitrite oxidizers.

Some heterotrophic bacteria such as Arthrobacter sp. have been reported to demonstrate some nitrifying activity but this does not seem to make a significant contribution to nitrification activities in soil and water.

The sanitary engineer is interested in these processes since they contribute to the ultimate biological oxygen demand, they occur to a varying extent during the biological treatment of wastewaters and are important precursor steps for denitrification processes. The sanitarian is interested in these processes since high concentrations of nitrates or nitrites in drinking water can cause a serious disease (methaemoglobinemia) in infants.

The process of ammonification-nitrification can best be described as metabiotic, i.e. the activity of one organism relying upon the previous activity of another. In this experiment the production of ammonia from an organic substrate (urea) will be observed. This release of ammonia will allow the ammonia oxidizers to grow producing NO_2^- which is in turn oxidized by Nitrobacter or Nitrocystis to NO_3^-. This is a dynamic process so that there should be some overlap during these three metabolic phases.

Procedure

1. Inoculate a 250 mℓ flask containing 100 mℓ of ammonification-nitrification medium (ANM). A second uninoculated flask of ANM should serve as a control. To the first flask, add 5 mℓ of secondary sewage effluent (preferably from an activated sludge plant) or 1 g garden soil. The flasks should be plugged with cotton and incubated under aerobic conditions at 25 C on a rotary shaker.

2. Over a period of 7-14 days, remove 5-10 mℓ samples of material from each flask and analyze for the presence of ammonia, nitrite and nitrate. Sample every day for the first 2 days and every other day after th[illegible]. When the amount of ammonia begins to decrease in the test flask, add calcium carbonate so that a final concentration of 1 g $CaCO_3$/100 mℓ medium is attained. If quantitative analyses cannot be performed at the sampling times, use a spot plate and Nessler reagent to determine the relative concentration of ammonia.
3. To demonstrate the activities of autotrophic ammonia oxidizers, inoculate 100 mℓ of Ammonia Mineral Medium (AMM) with 5 mℓ of an effluent from a nitrifying secondary treatment plant or with 1 g soil and incubate on a shaker at 25 C. Remove a small sample (1-2 mℓ) every other day for 7-14 days and using a spot plate qualitatively determine the relative amounts of ammonia, nitrite and nitrate present.
4. Using the same procedure as in step 3 above, inoculate flasks containing Nitrite Mineral Medium (NMM). This will demonstrate the activity of those autotrophic bacteria which oxidize nitrite to nitrate.
5. If time does not permit a quantitative chemical analysis to be performed after each sampling time, the collected samples may be acidified and frozen in sealed (capped) culture tubes and analyzed at a more convenient time.

Results

1. Record all data in a tabular form showing concentration and/or relative amounts of the various forms of nitrogen (NH_3, NO_2^-, NO_3^-)

with time. For the metabiotic experiment, plot the above chemical concentrations against time.

Apparatus

Erlenmeyer flasks, 250 mℓ

Porcelain spot plates

Rotary shaker

Reagents and Media

1. Calcium Carbonate, solid $CaCO_3$

2. Ammonificaition-Nitrification Medium (ANM)

Urea	0.35 g
KH_2PO_4	0.10 g
NaCl	0.30 g
$MgSO_4 \cdot 6H_2O$	0.14 g
$FeSO_4 \cdot 6H_2O$	0.03 g
Na_2HPO_4	0.1 g
Yeast extract	0.1 g
Distilled water	1000 mℓ

Filter sterilize,

3. Ammonia Mineral Medium (AMM)

$(NH_4)_2SO_4$	0.66 g
KH_2PO_4	0.14 g
NaCl	0.30 g
$MgSO_4 \cdot 6H_2O$	0.14 g
$FeSO_4 \cdot 6H_2O$	0.03 g
$CaCO_3$	10.0 g
Distilled water	1000 mℓ

Filter sterilize

4. Nitrite Mineral Medium (NMM)

   Same composition as ANM but substitute 0.5 g $NaNO_2$ for $(NH_4)_2SO_4$

5. Garden soil

6. Secondary sewage effluent

7. Secondary effluent from nitrifying secondary sewage treatment plant

Analytical Methods

1. Quantitative methods for the analysis of ammonia, nitrate and nitrite can be found in Reference 1, p. 412, 429 and 435.

2. Qualitative methods for the detection of ammonia, nitrate and nitrite are as follows:

   a. Ammonia can be detected by adding a few drops of culture to a spot plate and subsequently adding a few drops of Nessler reagent. An orange color indicates the presence of ammonia. Nessler reagent is prepared as described in Reference 1, p. 412.

   b. Nitrite can be detected using Griess-Llosavy reagents prepared as follows:

      (i) Sulfanilic acid reagent - to 300 mℓ of glacial acetic acid add to 0.5 g sulfanilic acid and mix. Add 100 mℓ of distilled water.

      (ii) α-Naphthylamine reagent - dissolve 0.1 g of α-naphthylamine in 100 mℓ of boiling distilled water. When cool add 30 mℓ glacial acetic acid.

      To a few drops of culture material add equal small volumes of the above two reagents. The development of a pink color indicated the presence of nitrite.

c. Nitrate can be detected by adding a small amount of zinc dust to a few drops of culture material in a spot plate. Wait several minutes then add equal portions of the Griess-Llosavy reagents (see Step 26 above). Compare the color produced with that developed when no zinc is added. If the color is darker in the former case then nitrates are present.

Note to Instructor: In this experiment the nitrification results may be accelerated by giving the student material from enrichment cultures previously prepared as in Step 3 of the Procedure. In this instance the inocula used would be 1 mℓ of enrichment medium rather than secondary effluent or soil.

EXPERIMENT I-D-3

DENITRIFICATION

Purpose

The purpose of this experiment is to demonstrate bacterial denitrification through the reduction of nitrate to nitrogen gas.

References

1. Alexander. M. Microbial Ecology, John Wiley and Sons, N.Y., 1971.
2. McElroy, W.D. and B. Glass. Inorganic Nitrogen Metabolism, a Symposium, John Hopkins Press, Baltimore, 1956.
3. Standard Methods for the Examination of Water and Wastewater, 14th ed. American Public Health Association, pp. 412, 429 and 434, 1976.
4. Delwiche, C.C. and B.A. Bryan "Denitrification" Ann. Rev. Microbiol, 30:241, 1976.

Introduction

Denitrification is an important aspect of the nitrogen cycle. In this process a number of heterotrophic bacteria are able, under anoxic conditions, to reduce nitrate and nitrite to nitrogen gas. A general equation for this reaction is as follows:

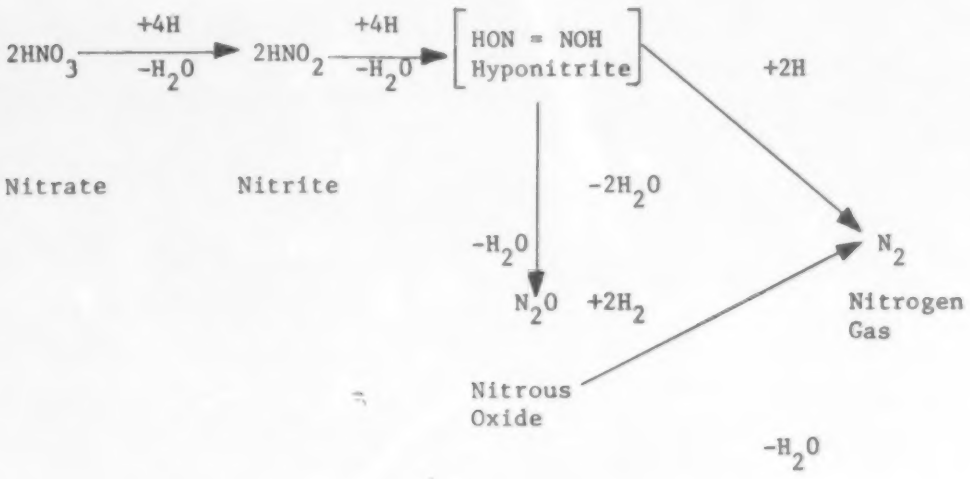
$$C_6H_{12}O_6 + 8\ NO_3^- \rightarrow 6CO_2 - 4N_2 + 8\ OH^- + 2H_2O$$

The bacteria involved in this process are basically aerobic; however, in the absence of oxygen they can grow using nitrate as the electron acceptor

rather than oxygen. This process is often referred to as a nitrate respiration. If oxygen is present, the reaction would be:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$$

Thus, although denitrification only occurs under anoxic conditions, it is strikingly similar to aerobic respiration. A suggested metabolic pathway is as follows:



As seen from this equation, nitrous oxide (N_2O) may be produced in small amounts in addition to nitrogen gas. Indeed, some authors feel that N_2O is the direct precursor of N_2.

A variety of bacterial species belonging to the genera Pseudomonas (for example, P. aeruginosa), Achromobacter, Bacillus, and Micrococcus will anaerobically reduce nitrogen in this fashion. There is at least one known chemoautotroph, Thiobacillus denitrificans which will denitrify under anaerobic conditions. Under aerobic conditions T. denitrificans uses oxygen as its electron acceptor.

Denitrification is of great importance in both agriculture and wastewater treatment. In the soil, denitrification may result in a loss

of fertility so that normally the farmer tries to inhibit the process. In wastewater treatment denitrification can be used as a biological nitrogen removal process. The degradation of an added organic compound (e.g. methanol), the sewage organics themselves or the endogenous respiration of the activated sludge are used as sources of reducing power to reduce nitrate to nitrogen gas.

Procedure

1. Prepare three 125 mℓ sterile flasks (A,B & C) containing 100 mℓ of minimal denitrification medium (MDM). These flasks and their contents should be maintained at between 25-30 C prior to use so that their dissolved gas content will be the same as that at the temperature of the experiment.
2. Fit the flask with a sterile one-holed stopper through which a short section of glass tubing has been inserted and to which a length of rubber tubing passing through an ascarite tube has been attached. The growth unit should be attached to a gas collecting device as illustrated in Figure I-D-I.

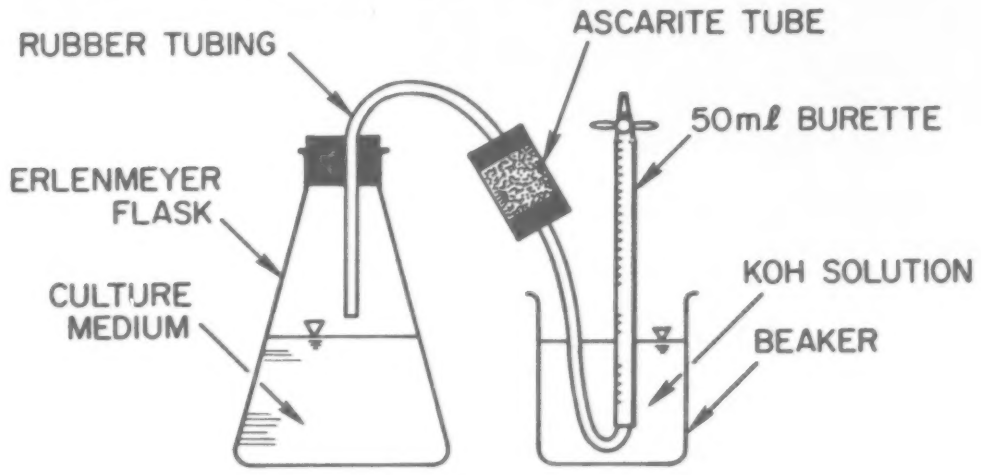


FIGURE I-D-I. APPARATUS FOR DETERMINING DENITRIFICATION

3. Fill the burette to the 45 mℓ mark with confining fluid (5% KOH) by opening the stopcock and carefully drawing up the fluid with a vacuum. The KOH will absorb CO_2 produced.

4. Flush out each flask aseptically with nitrogen gas. This may be accomplished by attaching a hose to a long hypodermic needle into the hub of which has been placed non-adsorbent cotton. The hose with prepared needle can be autoclaved. To use, attach to a nitrogen gas cylinder, flame the needle and insert under the stopper. Allow to flush with gas for a few minutes to replace the air with nitrogen.

5. Treat the three flasks as follows:

   FLASK A - Add 1 mℓ of a 24-hr culture of Pseudomonas aeruginosa, grown in MDM.

   FLASK B - Add 1 g garden soil or pond sediment.

   Flasks A and B should be inoculated while nitrogen gas is flowing into unit.

   FLASK C - This will act as control and as a thermobarometer. Do not inoculate.

6. Securely attach (stopper) each flask to its gas collector and incubate each growth unit at 25-30 C in a hot air incubator. After about 30 min incubation record the burette reading. This will be the Zero time reading.

7. Observe and record gas production over a period of several days and when gas production appears to have ceased make the following measurements:

   a. The total volume of gas produced in flasks A and B minus any gas produced in the control unit.

b. Determine the concentrations of NO_3-N, NO_2-N, and NH_4-N in an 0.45 μm membrane filtrate of each of the flasks including the control unit. Use a tared filter for Flask A.

c. Dry the residue collected on the membrane filter from Flask A in a hot-air oven at 80 C for 12 hr (overnight) then cool and weigh. This will be an estimate of biomass produced.

Data Analysis

1. Record the total volume of gas produced in Flasks A and B. Assume all the gas produced is nitrogen. Record the amounts of NO_3-N, NO_2-N and NH_4-N remaining in each flask. From Flask A, record the mg dry weight of bacteria formed.
2. Using the data from Flask A, perform a nitrogen balance in which you account for the amount of nitrate reduced to nitrogen gas, the amount of nitrate used in biosynthesis (assume that the dry bacterial cells contain 15% nitrogen) and the amount of NO_3-N reduced to nitrite and ammonia.
3. Compare the results of gas production and changes in NO_3-N, NO_2-N and NH_3-N obtained using a well-defined system (Flask A) with those obtained from the soil sample (Flask B). From your data estimate the biomass produced in the soil sample and discuss any complications found in arriving at this estimate.
4. How do your results fit with theoretical or expected results?

Apparatus

Growth units with gas collection device: See Figure I-D-I

Nitrogen cylinder with 2-stage pressure regulator

Hypodermic needle, 2 in. length

Membrane filter apparatus and membrane filters

Hot air incubator, 25-30 C

Oven, 80 C

Reagents

1. Minimal Denitrification Medium (MDM)

KNO_3	1 g
K_2HPO_4	0.5 g
$CaCl_2 \cdot 6H_2O$	0.5 g
Glucose	10 g
Distilled water	1000 mℓ

2. Garden soil or pond mud

3. KOH, 5% w/v.

Analytical Methods

NH_3-N - Determine by Direct Nesslerization (Reference 3, p. 412) Use Rochelle salt and EDTA reagents to prevent precipitate formation.

NO_3-N - Determine by the Chromotropic Acid Method (Reference 3, p. 429)

NO_2-N - Determine by Diazotization Method (Reference 3, p. 434).

EXPERIMENT I-E-1

SURVIVAL OF NON-INDIGENOUS BACTERIA IN NATURAL WATERS

Purpose

This set of experiments demonstrates the survival characteristics of two bacterial species not normally found in uncontaminated natural water, a gram-positive coccus, Staphylococcus aureus and a gram-negative coliform, Escherichia coli. In the first part of the experiment, the role of other natural water microorganisms in determining E. coli survival is illustrated. Isolation of predators on E. coli is also carried out. The effect of seawater on S. aureus is illustrated in the second part of the experiment.

References

1. Orlob, G.T. "Viability of Sewage Bacteria in Seawater", Sewage and Ind. Wastes, 28:1147, 1956.
2. Mitchell, R. "Destruction of Bacteria and Viruses in Seawater," J.S.E.D., ASCE., 97(SA4): 425, 1971.
3. Saz, A.K., Watson, S., Brown, S.R., Cowry, D.L. "Antimicrobial Activity of Marine Water", Limn. Oceanog. 8:63, 1963.
4. Enzinger, R.M. and R.C. Cooper. "Role of Bacteria and Protozoa in the Removal of Escherichia coli from Estuarine Waters", Appl. and Environ. Microbiol. 31:758, 1976.

Introduction

Transmission of disease by the water route is a primary health

concern. Escherichia coli is used as an indicator organism for fecal pollution, and is also a useful model of the survival of enteric organisms in natural bodies of water.

If an aquatic ecosystem is perturbed, for example, by a heavy dose of coliforms from a sewage outfall, the natural balance of microorganisms is eventually restored. In principle, this would be true for any non-indigenous population of bacteria because of their inability to withstand the new environmental pressures to which they are subjected.

Both physical-chemical and biological factors are responsible for the general decline of non-indigenous bacterial populations. Among the physical-chemical factors are:

1. Dilution - the monitoring of ocean outfalls has indicated this to be only partially responsible;
2. Adsorption and Sedimentation - this is significant in waters of high turbidity;
3. Temperature - the temperatures found in most natural waters cannot support the growth of enteric organisms, although low temperatures may prolong their survival;
4. High Concentration of Salinity and Cations - this inhibits the growth of non-indigenous species in the marine environment, in particular the gram-positive organisms.

Some of the biological factors considered to have an effect are:

1. Nutrient Conditions - enteric pathogens are fastidious organisms with relatively complex nutritional requirements and are ineffective competitors for the relatively low concentrations of organic carbon (5-25 mg/ℓ) in non-eutrophic waters;

2. Biogenic Toxins - produced by the indigenous population of bacteria, phytoplankton and macroalgae, especially in marine waters;

3. Predators - protozoa are considered very effective in keeping bacterial numbers low in natural waters, and the predatory bacteria Bdellovibrio sp. may also play a role.

A. Effect of Biological Factors on E. coli Survival

Procedure

1. Melt L-EMB agar and hold at 50 C.

2. Dispense 120 mℓ water sample into two 250 mℓ erlenmeyer flasks. Autoclave one of the flasks and label CONTROL.

3. Add 0.1 mℓ of concentrated E. coli suspension to both flasks.

4. Take "Day 0" assay for E. coli on both control and active sample as follows:

   a. With sterile 1.0 mℓ pipettes and sterile dilution blanks, sample 1.0 mℓ and make 10-fold dilutions to 10^{-7}

   b. Dispense the following into sterile petri plates (in duplicate)

      0.1 mℓ of the 10^{-7} dilution

      1.0 mℓ of the 10^{-7} dilution

      1.0 mℓ of the 10^{-6} dilution

   c. Pour approximately 15-20 mℓ L-EMB into plates and swirl to mix. After agar solidifies, incubate, inverted at 35 C for 24-48 hr. Count colonies and determine number of E. coli/mℓ of sample.

5. Make subsequent E. coli assays according to the following schedule:

ACTIVE SAMPLE

Day	Dilution						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
0	X	X	X	X	X	XY	XY*
1	X	X	X	X	X	XY	XY
2	X	X	X	XY	XY	XY	
3	X	X	XY	XY	XY		
4	X	XY	XY	XY	XY		
5	X	XY	XY	XY	XY		
6	XY	XY	XY				
7	XY**	XY					

CONTROL

Day	Dilution						
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
0	X	X	X	X	X	XY	XY*
4	X	X	X	X	X	XY	XY*
7	X	X	X	X	X	XY	XY*

*Dispense 0.1 mℓ of 10^{-7} dilution into plate marked 10^{-8} dilution.

**Dispense 1.0 mℓ sample directly onto plate marked 100 dilution.

^{X}Dilutions to be carried out.

^{Y}Pour plate to be made.

6. To observe predators, make double layer (DL) plates on Days 3 or 4 as follows:

   a. Melt 2 soft agar tubes by boiling them in a beaker of water.

   b. Hold at 50 C.

   c. Add 0.5 mℓ concentrated E. coli suspension to both tubes.

   d. Add 0.1 mℓ sample to first tube.

      Add 0.1 mℓ sample to second tube.

   e. Gently swirl and pour on to plain agar plates. Incubate at room temperature for 2-7 days (do not invert).

   f. Check periodically for clear plaques in the E. coli lawn.

   g. When plaques appear, transfer a small piece of plaque material to a drop of water on a slide using a sterile loop. Press the cover slip down on the material to flatten it and observe microscopically under 45X and 100X.

Data Analysis

1. Tabulate and plot the data graphically in the form: log no. E. coli (vertical axis) vs. days (horizontal axis). Include control on same graph.
2. Estimate the numbers of predators in the sample by counting the plaques in the DL plates. Describe the microscopic observations made.
3. Considering how the controls were prepared, what factors affecting bacterial survival are being demonstrated in this exercise?
4. What kind of organisms were observed to affect E. coli numbers?
5. Design an additional experiment which would substantiate the effect of microbial predators on E. coli survival.

Apparatus

Incubator, 35 C

Compound microscope, with 45X and 100X objectives

Slides and coverslips

250 mℓ erlenmeyer flasks, 2 per student group

Sterile petri plates, 60 per student group

Sterile 1.0 mℓ pipettes, 75 per student group

Water bath at 50 C to hold liquid agar

Rotary shaker

Reagents

Levine's Eosin Methylene Blue Agar (L-EMB), follow manufacturer's instructions, autoclave at 121 C for 15 min; 1200 mℓ per student group.

Pre-poured plain agar plates; dissolve 15 g plain agar in 1ℓ lake, river or seawater, then autoclave and pour; 4 per student group.

Soft agar tubes: Dissolve 7.5 g plain agar in 1ℓ filtered lake, river or seawater, heat to dissolve, dispense 4 mℓ per test tube and autoclave; 4 per student group.

Dilution blanks, 9 mℓ portions: dissolve 8.5 g KH_2PO_4, 21.75 g K_2HPO_4 33.4 g $Na_2HPO_4 \cdot 7H_2O$ in 500 mℓ distilled water and dilute to 1ℓ. This should be pH 7.2. Add 1 mℓ of this solution at the rate of 1mℓ/ℓ distilled water to prepare dilution blank water; apportion into 9 mℓ portions in test tubes; sterilize by autoclaving; 50 per student group.

Washed, concentrated suspension of E. coli: Prepare 2ℓ sterile nutrient broth, inoculate E. coli and incubate on shaker for 48 hr. Centrifuge cells in sterile cups and wash once with sterile buffer.

Resuspend in 20 mℓ sterile buffer and refrigerate. This is sufficient for 10 student groups.

Water sample from lake, river or ocean, freshly collected.

B. The Effect of Sea Water on the Survival of S. aureus (Reference 3)

Procedure

1. Melt nutrient agar and hold at 50 C.
2. Filter approximately 200 mℓ seawater (SW) through an 0.22 μm membrane filter and decant 120 mℓ of the filtrate into a sterile 250 mℓ erlenmeyer flask.
3. Decant 120 mℓ sterile buffer into a sterile erlenmeyer flask and label BLANK.
4. Add 0.1 mℓ of concentrated S. aureus suspension to both samples. Take "Day 0" assays for both Blank and Active sample, using nutrient agar for pour plates.
5. Follow the same sampling and dilution protocol as for Experiment I-E-1A.

Data Analysis

1. Tabulate data and plot the data in the form: log no. S. aureus vs. days. Include the control on the same graph.
2. Considering the procedure followed, what kind of factors are found to affect S. aureus survival?

Apparatus

Millipore filtration apparatus and 0.22 μm membrane filters, sterile Erlenmeyer flasks, 250 mℓ, sterile; 2 per student group

Incubator, 35 C

Sterile petri plates, 60 per student group

Sterile 1.0 mℓ pipettes, 75 per student group

Water bath at 50 C to hold liquid agar.

Reagents

Nutrient agar: prepare according to manufacturers directions, 12000 mℓ per student group.

Washed concentrated suspension of S. aureus prepared as for E. coli in Experiment I-E-1A.

Freshly sampled seawater from the vicinity of a sewage discharge.

Dilution blanks, 9 mℓ: See Experiment I-E-1A, 50 per student group.

Buffer water, sterile: Same composition as dilution blanks, 150 mℓ per student group.

EXPERIMENT I-F-1

MEASUREMENT OF PRIMARY PRODUCTIVITY

Purpose

In this experiment two techniques for the measurement of primary productivity are presented. These are the uptake of ^{14}C labelled inorganic carbon and the production of oxygen. While both of the techniques can be used for laboratory and *in situ* measurement of primary productivity the ^{14}C method is described for field measurements and the oxygen production method is described as a laboratory technique.

References

1. Standard Methods for the Examination of Water and Wastewater, 14th ed., American Public Health Assciation, p. 441, 1976.
2. Steemann-Nielsen, E. J. Consiel int. Explor. Mer, 18:117, 1952.
3. Watt, W.D. Limnol. Oceanogr., 10:298, 1965.
4. Goldman, C.R. J. Consiel int. Explor. Mer., 32:172, 1968.

Introduction

In the overall photosynthetic reaction CO_2 is reduced and incorporated into organic matter with the accompanying oxidation of water to oxygen. These gross reactions in green plants including algae can be expressed as:

$$CO_2 + H_2O + nh\nu \xrightarrow[\text{enzymes}]{\text{chlorophyll}} \text{"}CH_2O\text{"} + O_2$$

where

"CH_2O" is a representation of an organic compound at the oxidation state of a carbohydrate

and

$nh\nu$ represents the utilization of light energy for the photolysis of water.

Since plankton are usually the primary producers of organic matter in natural waters the measurement of the rate of their photosynthetic reaction may be regarded as an index of the primary productivity of the water. Two possibilities for measuring the rate of this reaction present themselves: these are the uptake of inorganic carbon and the production of oxygen. Inorganic carbon uptake can be assayed by allowing the photosynthetic reaction to proceed for a given time period in the presence of ^{14}C labelled inorganic carbon ($^{14}CO_3^{2-}$ or $H^{14}CO_3^-$) then determining the amount of ^{14}C incorporated into the photosynthetic microorganisms. Oxygen production can be determined by measuring the change in dissolved oxygen concentration of a water incubated for a known period of time. In both of the techniques it is necessary to correct for non-photosynthetic activities such as respiration that consume oxygen thus:

$$"CH_2O" + O_2 \xrightarrow[\text{enzymes}]{} H_2O + CO_2$$

and activities that incorporate $^{14}CO_2$ into organic compounds through non-photosynthetic reactions such as the Krebs cycle.

The corrction for such reactions is usually performed by incubating a sample in the absence of light (a so-called dark bottle) and using the ^{14}C uptake or oxygen uptake of this bottle to correct for non-photosynthetic reactions.

When using ^{14}C the dark bottle measures more than "background" incorporation of ^{14}C. First, the use of a dark bottle may stimulate or repress background ^{14}C uptake which occurs in the light (photo-inhibition

and photo-repression of respiration). Secondly, algae suddenly placed in the dark often excrete large amounts of organic carbon. Photosynthetic inhibitors such as dichloromethyl urea (DCMU) have been used in light bottles to give a better background but the effects of this inhibition on other processes are not known. Consequently, it is preferable in measuring primary productivity using the ^{14}C technique for a series of measurements to express ^{14}C (light) and ^{14}C (dark) graphically so that anomalous dark bottle responses can be modified by later examination of the data.

A. Field (or in situ) Measurement of Primary Productivity Using ^{14}C

Note: This experiment may be performed equally well using the oxygen production technique.

1. Using either an underwater photometer or a Secchi disk, determine the depth at which there is a one percent transmission of incident light. This depth is approximately 2.5 times the Secchi disc depth. This depth represents the limit of the euphotic zone, the zone in which photosynthesis occurs.
2. Make up a suspension line that has attachments at 6 spaces, evenly - placed in the euphotic zone, and one attachment below the photic zone.
3. Prepare 14 light bottles and 7 dark bottles each with a 125 mℓ capacity.
4. Collect water samples from each of the 7 depths established above using either an opaque sampler or a pump whose intake can be located at the 7 depths established above.

5. Immediately transfer the collected sample from each depth to two light and one dark bottle filling them to the brim and stoppering tightly. Keep all bottles and water out of sunlight and in as deep a shade as possible at all times.

6. Collect sufficient water samples for the determination of total dissolved inorganic carbon. If this determination is to be performed by calculation from an alkalinity measurement, immediately measure the pH of the samples.

7. After all bottles have been filled, add ^{14}C solution to each. Break a 10 mℓ ampule containing ^{14}C solution and using an automatic syringe with a long (10 cm) needle transfer between 0.1-1 mℓ ^{14}C solution into the bottom of the bottles. The amount of ^{14}C solution (and the strength of the solution used) is determined by the productivity of the water and the depth at which the samples are incubated. Typically, for highly productive waters 0.1 mℓ of a 1 μcurie/mℓ ^{14}C solution is sufficient while for oligotrophic waters up to 1.0 mℓ of a 5 μcurie/mℓ ^{14}C solution may be necessary. Stopper the bottles firmly immediately after adding the ^{14}C solution.

8. Attach two light and one dark bottle to each of the seven depths and lower into the water. Incubate for 2-4 hr preferably during the period around noon (e.g. 1100-1300, 1000-1400).

9. After incubation remove the bottles rapidly. Keep in the dark and as rapidly as possible filter the entire contents of the bottles (or in productive situations a well-mixed aliquot of 25-50 mℓ) through filters of 0.45 μm pore size using a Millipore filtration apparatus (if possible with a stainless steel membrane supporting

screen). Identify all filters before filtration by marking with a ball point pen or with a pencil.

10. Place filters in individual desiccators to transport to the laboratory. To ensure that filters remain flat during transport and drying, place on them a small piece of metal or glass tube cut into a ring that is just larger than the filtered area but smaller than the filter itself.

11. Determine ^{14}C counts per minute (cpm) on each dried filter using either a planchet Geiger-Müller (G-M) counter or a liquid scintillation counter. If a liquid scintillation counter is used, place the filters directly in the liquid scintillation "cocktail". At least 1000 counts should be registered for each filter in duplicate or triplicate. Compute the average cpm from these counts for each filter and from background counts performed in the same fashion.

12. In order to convert cpm data to amount of carbon fixed photosynthetically it is necessary to employ the following equation:

$$\frac{\text{Light bottle filter, cpm/m}\ell - \text{Dark bottle filter, cpm/m}\ell}{\text{cpm added/m}\ell} \times \text{Dissolved Inorganic Carbon, mg/m}^3$$

$$\times \text{ Correction factor} = \frac{\text{mg Carbon Fixed}}{\text{m}^3, \text{ hr}}$$

In this equation there are three terms that need to be determined. Their measurement/calculation is given below.

a. <u>cpm added/mℓ</u>

This is the activity in cpm/mℓ of bottle volume of the original aliquot of ^{14}C added. This is best determined by precipitating all the $^{14}CO_3^{2-}$ in replicate ampules with an excess of $BaCl_2$. The $BaCO_3$ so formed is filtered and its activity measured either by:

(i) A gas phase counter (100% efficient) or

(ii) By G-M counting of several filters each containing different but known amounts of $BaCO_3$ precipitate. From the cpm obtained a plot (similar to Figure I-F-I) of precipitate quantity in $\mu g/cm^2$ vs. cpm is prepared and the cpm at infinite precipitate thickness is estimated by extrapolation.

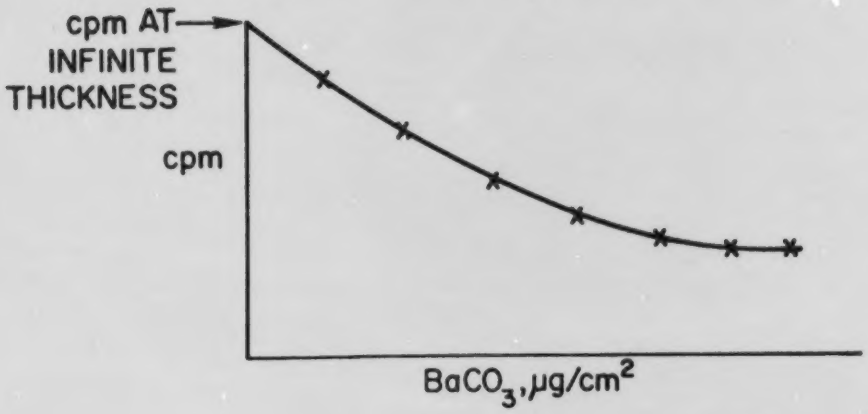


FIGURE I-F-I. ^{14}C cpm VS. AMOUNT OF $BaCO_3$ PRECIPITATED

b. Dissolved Inorganic Carbon

This may be determined by a carbon analyzer (e.g. Beckman Model 900) with an infrared detector or from a combination of initial pH measurement and an alkalinity titration using standard acid and a methyl orange indicator or a pH meter. Dissolved inorganic carbon is computed as:

$$\text{Dissolved Inorganic Carbon, mg/}\ell = \frac{\text{Alkalinity}}{\alpha_1 + 2\alpha_2 + [OH^-] - [H^+]}$$

where

$$\alpha_1 = \frac{1}{\frac{[H^+]}{K_1} + 1 + \frac{K_2}{[H^+]}}$$

$$\alpha_2 = \frac{1}{\frac{[H^+]^2}{K_1K_2} + \frac{[H^+]}{K_2} + 1}$$

K_1 = First ionization constant of H_2CO_3

K_2 = Second ionization constant of H_2CO_3

Alkalinity in uhits of meq/ℓ.

This relationship is only legitimate for closed systems not in contact with a carbonate-bearing precipitate and if the carbonate system is the only significant buffering system. This is true of most fresh waters but in marine and some estuarine waters contributions from constituents such as borate may be significant.

c. Correction Factor

The correction factor includes the following terms:

$$\frac{1}{\text{Efficiency of Counter, \%}} \times \frac{1}{\text{hr of incubation}} \quad \frac{\text{mℓ}}{\text{m}^3}$$

The efficiency of the ocunter is measured by counting a standard source.

Data Analysis

1. Plot the primary productivity $\frac{\text{mg C}}{\text{m}^3, \text{ hr}}$ vs. depth (m) for the euphotic zone. Explain the shape of this curve.

2. If primary productivity were to be measured at the periods 0600-0800, 1000-1200, 1400-1600, 1800-2000 what type of relationship would you expect between these values.

3. It is known that some algae secrete some soluble organic compounds containing ^{14}C during incubation. How would you correct primary productivity measurements for this type of phenomenon?

Apparatus

Pyrex or borosilicate glass bottles: 125 mℓ capacity; ground glass stopper; 24 per student group.

One third of these bottles are made into "dark bottles" by taping with black tape, spray-painting bottle and stopper with matt black paint and covering with aluminum foil. All bottles should be fitted with spring clips for rapid attachment of the bottles to a line.

Automatic syringe: capacity 0.1 to 1.0 mℓ with approximately 10 cm long needle.

Floats, anchor and line with loop at predetermined depths.

A β-counting device: either a planchet G-M counter or a scintillation counter, or a gas phase β-counter.

Membrane filters: pore size of approximately 0.45 μm.

Filter apparatus: Millipore with stainless steel support for membrane; top of filter apparatus should be calibrated to known volumes or be capable of taking all 125 mℓ of the sample.

A light-weight light box with compartments for 24 bottles and a black cloth to cover it.

Small pieces of metal/glass tube cut into rings to be just larger than the filtered area but smaller than the filter itself.

Pianchets: large enough to hold a 25 µm diam. membrane iflter

Light meter: with underwater detector and deck cell

Secchi disc

Carbon analyzer: Beckman Model 900 or equivalent

Burette: 25 mℓ capacity

pH meter

Individual desiccators: 2 oz wide-mount, screwcap jars containing indicating silica gel and support for filter.

Reagents

Glass ampules: 10 mℓ capacity containing $Na_2{}^{14}CO_3$ or $NaH^{14}CO_3$ solution with activity in the range 1-5 µcuries/mℓ

Liquid scintillation cocktail: Commercially available

Sulfuric acid: 0.01 N

Barium chloride solution: 5% w/v

Methyl orange indicator solution: 0.5% in 95% ethanol

B. Measurement of Primary Productivity Using Oxygen Production

Note: While the procedure given is a laboratory technique it may be used in situ employing the same general procedures as descirbed in the ^{14}C experiment.

Procedure

1. This experiment may be carried out in various degrees of sophistication that range from the simple incubation of a natural water at one

light intensity in the laboratory to the sampling at various depths in the photic zone and the incubation of samples at several light intensities corresponding to those at the depths of sampling. In this procedure the simplest experiment is outlined since the ^{14}C procedure examines the more complex field assessment of primary productivity. This experiment is designed to serve more as a demonstration of technique rather than an assessment of actual productivity.

2. Obtain a sample of natural water from the photic zone of a lake or stream. Transport in a darkened condition to the laboratory.
3. As rapidly as possible and with the minimum of aeration during pouring, transfer the water sample to five 125 mℓ capacity light bottles and two 125 mℓ capacity dark bottles.
4. Stopper all the bottles immediately excluding all air bubbles. Commence the determination of the dissolved oxygen content of two of the light bottles by immdiately adding the first two Winkler reagents ($MnSO_4$ solution and alkaline KI + azide) to them and shaking.
5. Incubate 3 light bottles and 2 dark bottles in a water incubator maintained at the temperature of the natural water. The bottles should be incubated laying on their sides and should be placed in the incubator in such a way that they do not cast shadows on each other. The incubator should be painted white on the interior and should be illuminated evenly with a combination of cool-white and grow-lux fluorescent tubes to give an incident light intensity of approximately 2500 foot-candles at the surface of the incubated

bottles. Measure this intensity with a light meter.

6. If it is desired to attempt a simulation of an in situ experiment several incubation tanks may be used in which the light intensity at various depths in the photic zone is duplicated in the various incubators using a neutral screen such as a black cloth or muslin of variable thickness and weave. In such an experiment the water samples should be collected from the individual depths as in the ^{14}C experiment, and the light intensity must be measured at the time of sampling. It may be useful to establish incubators at 100%, 50%, 25% and 10% of the incident illumination.
7. The bottles should be incubated for a period of about 4-6 hr depending on the degree of illumination and the productivity of the water.
8. After incubation remove all of the bottles from the incubator and immediately determine their dissolved oxygen content using the azide modification of the Winkler (Iodometric) Method (Reference 1).

Data Analysis

1. Determine productivity of the water in terms of mg O_2/ℓ-hr using the following calculation:

$$\frac{DO_l + (DO_i - DO_d)}{t}$$

where

DO_l = dissolved oxygen in light bottle after incubation, mg/ℓ

DO_i = initial dissolved oxygen prior to incubation, mg/ℓ

DO_d = dissolved oxygen in dark bottle after incubation, mg/ℓ

t = time of incubation, hr

Apparatus

Pyrex or borosilicate glass bottles for light and dark bottles:

125 mℓ capacity

Dark bottles prepared as for ^{14}C experiment (Experiment I-F-1A).

Light meter with underwater detector.

Illuminated incubation tank or tanks as described in procedure.

Burette, 25 mℓ capacity.

Neutral filters as required to control light intensity.

Reagents

Reagents for determination of dissoved oxygen: azide modification of Winkler (Iodometric) Method (Reference 1, page 441).

EXPERIMENT I-G-1

THE GROWTH OF MICROORGANISMS ON SURFACES

Purpose

To illustrate the characteristic growth of bacteria and other microorganisms on surfaces in natural waters and the changes observed in the surface community as a function of time.

References

1. Henrici, A.T. and Johnson, D.E. "Studies of the Freshwater Bacteria", J. Bact. 30:61, 1935.
2. Tyler, P. and Marshall, K.C. "Form and Function in Manganese Oxidixing Bacteria", Arch. Microbiol., 56:344, 1967.
3. Heukelekian, H. and Crosby, E.S. "Slime Formation in Sewage", Sewage Ind. Wastes, 26:206, 1956.
4. Zobell, C.E. "The Effect of Solid Surfaces Upon Bacterial Activity", J. Bact., 46:36, 1943.

Introduction

Microbial populations in lakes, streams and oceans are predominantly associated with surfaces and particulate matter. Some bacterial species have speicific morphological adaptations hy which they attach to surfaces. For example, Caulobacter and Hyphomicrobium form stalks, while the filamentous Beggiatoa has a holdfast cell (Reference 1). Other suface-associated forms are known to produce extracellular polysaccharides which aid in their adhesion.

The growth of bacteria on surfaces is thought to be influenced by the molecular adsorption of organic compounds on to surfaces. Particularly, in natural waters of low organic levels, a greater availability of nutrients would therefore be found on these surface for supporting growth (Reference 4). Consequently, although bacteria are colloidal in size and influenced by the same forces which govern colloid behavior, they are additionally influenced by nutrient levels and specific attachment mechanisms.

In addition to being a characteristic of natural microbial communities, surface growth has practical implications. Thus surface growths can be a nuisance since their massive occurrence in pipelines can cause decreased carrying capacity and anaerobic conditions which lead to taste and odor problems and stimulate the corrosion of iron and concrete (References 2 and 3). On the other hand, surface growth is used to an advantage in trickling filter treatment systems.

The simplest method to observe suface communities is with the submerged slide technique which was first used for the aquatic habitat by Henrici (Reference 1). Many surface-adapted forms such as Caulobacter, in fact, are very difficult to observed by any other procedure. As with other natural communities, a succession of microbial forms with time can be observed. Depending on the quality of the water sample, not only is there an increase in biomass, but different microorganism types appear and various group predominate at different times. Although many organisms are firmly fixed to the solid surface, others are loosely associated and are easily washed away. If the time course is long enough, the development of microcolonies of bacteria can also be observed.

As discussed in Experiment III-A-1 a microbial succession is a non-random event whereby the development of each microbial group is influenced by and dependent upon the conditions provided by the preceding population. Varying the environmental conditions such as nutrient level, temperature, light, etc. will alter the direction of the succession. This can be readily observed in the laboratory.

Procedure

1. Suspend 25 clean glass slides into a sample of lake or river water. If desired, student groups can treat their water sample differently, e.g. add a source of organic carbon, place in the light, elevate the temperature.
2. At daily intervals for 7 days, remove 3 slides. Wipe one side of each slide dry with a towel and then wipe the same side with ethanol.
3. Wash one slide gently, but firmly with a stream of filtered water from the same source as the water in which it was suspended. This procedure removes all organisms not firmly attached to the surface. Make a wet mount by placing a coverslip on the untouched surface.
4. With the remaining 2 slides, prepare a Gram's stain with one and make a wet mount with the other.
5. Observe all three slides with a phase contrast microscope under 10x and 100x objectives to examine both large and small organisms. Use of a Whipple micrometer grid helps greatly in estimating numbers of organisms.

   Note: Observations with the standard compound microscope are difficult on unstained samples. If no phase contrast microscope is

available and observations on unstained samples in unsatisfactory, eliminate Step 3, making Gram's stain on one slide and a simple Methylene Blue stain on the other.

Data Analysis and Questions

1. Tabulate your daily observations noting the different morphological types of organisms.
2. Note the time of appearance and relative numbers of unusual forms e.g. stalked bacteria, filamentous bacteria, fungi, protozoa, algae.
3. Note the relative proportion of gram positive and gram negative bacteria over the 7-day observation period.
4. What types of organisms are the initial colonizers (Days 1 and 2)? At what times did protozoa, fungi or algae first appear?
5. Describe the succession you observed. Discuss what characteristics may be of advantage to initial colonizers and suggest why later colonizers do not appear sooner.

Apparatus

Standard compound microscope

Phase contrast microscope

Whipple micrometer grid

Specimen dishes or small aquaria

Glass slides, 25 per student group

Nylon thread or fish line

Epoxy

Hand Tally

Glass rods

Reagents

Gram's stain set

Methylene Blue stain: Dissolve 5g Methylene Blue in 100 mℓ 95% ethyl alcohol, allow to stand overnight and then decant.

II. PUBLIC HEALTH ASPECTS

EXPERIMENT II-A-1

ENTERIC BACTERIAL PATHOGENS - ISOLATION OF

SALMONELLA AND SHIGELLA FROM SEWAGE

Purpose

To determine the presence of *Salmonella* and *Shigella* bacteria in wastewater and to introduce the student to the techniques used for the isolation of these bacteria from the environment.

References

1. Cooper, R.C. "Wastewater Management and Infectious Disease," *J. Environmental Health*, 37:217, 1974.
2. Kenner, B.A. and Clark, H.P. "Detection and Enumeration of *Salmonella* and *Pseudomonas aeruginosa*," *J. Water Pollution Control Federation*, 46:2163, 1974.
3. *Difco Manual*. 9th ed. Difco Laboratories, Michigan, 1966.
4. *BBL Manual of Products and Laboratory Procedures*. 5th ed. BBL Division of BioQuest, Maryland, 1968.

Introduction

Bacteria of the genera *Salmonella* and *Shigella* contain a wide variety of species which are pathogenic for man and animals. Their primary source is infected fecal matter, and they are transmitted by means of personal contact, contaminated food and contaminated water.

The *Salmonella* are the more common and include more than 1500 serotypes, among them the agent of typhoid fever (*S. typhosa*). The most commonly

encountered Salmonella cause acute gastroenteritis and, in the United States, this is usually S. typhimurium. In 1965, 18,000 people were infected through a contaminated water supply in Riverside, California.

Bacteria of the genus Shigella produce an intestinal disease in man and higher apes known as bacillary dysentery. There have been a number of reported outbreaks of waterborne Shigellosis in small communities in the United States. There are six recognized species, the most common being Sh. sonnei and Sh. flexneri.

The Shigella and Salmonella are gram-negative, enteric bacteria. They either fail to ferment lactose or do so at a very slow rate. They are classified on the basis of biochemical reactions and serological tests.

Although the organisms are present in sewage, they are difficult to recover. Not only are they present in relatively small numbers but they also tend to be outgrown by other types of bacteria when the sewage sample is cultured on ordinary media. In order to detect the Salmonella and Shigella, it is usually necessary to concentrate the sewage sample and then to use a series of special media designed to enrich and select for these two genera.

The sewage swab technique has been found to serve as a satisfactory mode of concentrating the sample. This procedure was developed in England where it was used to trace typhoid carriers. The swab is a length of gauze or cheesecloth folded over a square of cotton. The rectangle is sewn and a piece of twine is attached to one side. The device is then tied so that it is constantly immersed in the flow of sewage. After a period of time (5-7 days) it is removed and directly

placed into an enrichment broth. The inoculum, thus, is a concentrated sewage sample and contains organisms recovered during many hours of sewage flow.

Initial enrichment may be achieved using a number of different media. The most common are selenite, tetrathionate or dulcitol selenite (DSE) broth. These broths will inhibit the growth of coliform organisms and promote the development of Salmonella and Shigella. The latter broth will also encourage the growth of Pseudomonas sp. The enriched cultures are subsequently streaked onto solid (agar) media which are both selective and differential. The procedures used in this experiment will employ Salmonella-Shigella Agar (SS), Bismuth Sulfite Agar (BSA) and Xylose Lysine Desoxycholate Agar (XLD). The appearance of important colonies on these solid media is tabulated in Table II-A-I.

Typical isolated colonies are chosen from each agar medium and if necessary are restreaked on homologous media to achieve pure cultures. These isolates are further characterized by their reactions on Triple Sugar Iron Agar (TSI) and on Urea Agar. The expected reactions on these two media are shown in Table II-A-II.

TSI agar contains ferrous salts which combine with hydrogen sulfide to form a black precipitate of FeS. It also contains three sugars: lactose (10 g/ℓ), saccharose (10 g/ℓ), and dextrose (1 g/ℓ). Organisms which can utilize all three sugars ferment them, producing acid and changing the color of the indicator from red to yellow. The Salmonella and Shigella groups can use dextrose only. Their continued growth necessitates a breakdown of the peptones present. Protein hydrolysis releases ammonia, which raises the pH and the indicator becomes red.

Thus, the initial color (i.e., during less than 12 hr incubation) of the TSI tube containing Salmonella or Shigella is yellow; continued incubation results in the slants becoming red. Organisms fermenting the three sugars produce acid conditions and the indicator turns. Normally, sufficient acid is produced to limit further metabolism so that the yellow color persists.

Since Salmonella and Shigella do not ferment lactose or saccharose, the slant will not change color but the butt will become acid. Coliforms can ferment lactose and will turn the entire tube of agar yellow. Proteus which is not usually considered to be an enteric pathogen will frequently give a reaction similar to Salmonella and Shigella. Thus, another differential test, urease activity, is required for differentiation. Proteus is typically urease positive.

Possession of urease enables a bacterium to hydrolyze urea into carbon dioxide and ammonia, which raises the pH of the medium. The phenol red dye present in Urea Agar indicates this phenomenon by changing to a cerise color above pH 8.1. (See Table II-A-II).

This experiment will terminate after the Urea Agar and TSI reactions are noted and the results reported as a tentative diagnosis. Further differentiation of the isolates may be obtained by noting their ability to ferment various sugars with or without the concomitant production of gas. Serological tests are employed to further type the organisms.

TABLE II-A-I

COLONIAL APPEARANCE OF ENTERIC BACTERIA ON BSA, SS and XLD AGAR

Bacterial Group	Agar Medium		
	SS	BSA	XLD
Salmonella, Shigella, and Proteus.	Opaque, transparent or translucent uncolored colonies. Some Salmonella may produce colonies with black centers	S. typhosa produces round jet black colonies with a black metallic sheen. Other Salmonella, Shigella and Proteus colonies are flat or slightly raised green or brownish with depressed centers.	Salmonella produce red, black-centered colonies. Shigella colonies are usually all red.
Coliforms	Pink or red colonies; some may be large, white and mucoid.	Small black, brown or green colonies frequently with a silvery sheen.	Yellow colonies.

TABLE II-A-II

TYPICAL REACTIONS OF VARIOUS ENTERIC BACTERIA ON TSI AND UREA AGARS

Organism	TSI			Urea	
	Butt	Slant	H_2S	Butt	Slant
Shigella sp	A	NC	(-)	(-)	(-)
Salmonella sp	AG	NC	+	(-)	(-)
E. coli	AG	A	(-)	(-)	(-)
Enterobacter sp	AG	A	(-)	(-)	C
Proteus sp	AG*	A*	+*	C	C

A = Acid; G = Gas; NC = No change; (-) = No reaction

C = Cerise; * = Variable reaction.

Note: A few species of Salmonella and Shigella do not give typical reactions on TSI Agar.

Procedure

1. Suspend a gauze pad in sewage flow. Immerse for 4-7 days. A sanitary napkin makes a convenient swab.
2. Transfer the exposed swab to a beaker containing sufficient enrichment medium to cover the swab. Cover the beaker with sterile aluminum foil. Use one of the three enrichment broths (selenite, tetrathionate or DSE) as directed by your instructor. Incubate 18-24 hr at 35 C except for DSE which should be incubated at 40 C.
3. After incubation streak a loopful of enriched material onto one or two plates each of SS, BSA and XLD agar medium. Incubate at 35 C for 24 hr.

4. Choose appropriate colonies, as described in Table I-A-I and if not well isolated, streak onto homologous media and incubate 24 hr at 35 C. You should select at least five colonies from each medium and inoculate material from each colony onto TSI and Urea Agars. TSI should be inoculated with a straight needle; streak the slant, then stab the needle into the agar butt, taking care not to contact the glass sides; stop within 1/8 in. of the bottom of the tube. Inoculate Urea Agar with a needle also, but streak the culture only along the slant; DO NOT plunge the needle into the butt of the tube. Inocubate at 35 C. Observe the urea slants at 4 hr and at 24 hr. Observe the TSI slants at 12 hr and at 48 hr.

Results

1. Tabulate the reactions of each isolate on TSI and Urea Agar.
2. Calculate the frequency of selection of suspected Salmonella-Shigella from the differential agars.
3. Make a preliminary diagnosis of the type of enteric pathogen present in the wastewater studied. In presenting your results suggest a means of quantitating the number of pathogens present employing the type of methods used in this exercise.

Apparatus

Gauze swabs (sanitary napkins)

Sterile beaker: 1ℓ capacity with aluminum foil cover.

Reagents

Tetrathionate Broth (Difco or BBL)

Selenite Broth (Difco or BBL)

Dulcitol Selenite Broth

Proteose peptone,	4 g
Yeast extract,	1.5 g
Dulcitol,	4 g
Sodium Acid Selenite,	5 g
Na_2HPO_4,	1.25 g
KH_2PO_4,	1.25 g
Distilled water,	1000 mℓ

Place ingredients in sterile flask covered with foil and heat to near boiling until mixture is clear. No further sterilization is required.

Bismuth Sulfite Agar (Difco or BBL)

Salmonella-Shigella Agar (Difco or BBL)

Xylose lysine desoxycholate agar (BBL)

Triple sugar iron agar slants (Difco or BBL)

Urea Agar (Difco)

EXPERIMENT II-B-1

INDICATOR ORGANISMS - ENUMERATION OF COLIFORMS AND FECAL COLIFORMS BY THE MOST PROBABLE NUMBER (MPN) METHOD

Purpose

To determine the presence of coliforms and fecal coliforms in water and wastewater and to enumerate these indicator organisms using the most probable number (MPN) method.

References

1. Standard Methods for the Examination of Water and Wastewater, 14th ed. American Public Health Association, p. 920, 1976.
2. Cochran, W.G. "Estimation of Bacterial Density by Means of Most Probable Numbers," Biometrics, 6:105-116, 1950.

Introduction

There are a number of microbial diseases which can be transmitted by the water route. The causative microorganisms are usually not readily isolated from water because they are present in low numbers. Their enumeration in water samples is extremely difficult and tedious. Because of these difficulties, the microbiologist relies upon certain indicator organisms whose presence can be easily determined and quantitated, and whose occurrence is indicative of the presence of human or animal wastes which have the potential to contain disease-producing microorganisms. The most commonly used indicators are a group of bacteria classified as coliforms.

The coliform group of bacteria is defined as any gram negative, non-sporogenous, facultative anaerobic rod which ferments lactose with the production of gas. This definition encompasses a number of species of bacteria including Escherichia coli, Enterobacter aerogenes, a number of species and varieties of these two genera, and certain other bacteria. The coliform in the fecal meterial from man and other warm-blooded animals is predominantly E. coli. Certain strains of E. coli are known to be pathogenic to man.

The coliform test for the sanitary evaluation of water quality has been developing continously since before the turn of the century. Initially, it was felt that coliform organisms were solely fecal in origin, but it soon became apparent that there were other sources including soil and plants. Thus the coliform group is not as definitive an indicator of fecal contamination as would be desired. In recent years, however, an acceptable, utilitarian method has been developed for differentiating between fecal and non-fecal coliforms on a routine basis.

The test for coliforms utilizes special media which enrich and select for bacteria with the following characteristics:

(i) the ability to ferment lactose with the production of gas;

(ii) the ability to grow in the presence of certain anionic wetting agents; and

(iii) the ability to grow in the presence of specific concentrations of organic dyes such as Brilliant Green. These media will select for gram negative, non-sporeforming, lactose-fermenting rods.

The standard test for coliforms, as described in Reference 1, may be divided into three phases: (i) the presumptive test, (ii) the confirmed test, and (iii) the completed test. In presumptive tests, a known volume of water sample is inoculated into lactose broth and incubated for 24-48 hr at 35 C. The presence of bacteria which ferment lactose with the production of gas will be indicated by the presence of gas in tubes of broth containing gas-collecting vials. Since there are a number of gram positive organisms which can produce gas under these conditions, it is necessary to confirm these positive presumptive tubes by subculturing their contents into a medium containing lactose, oxgall (an anionic wetting agent) which will inhibit gram positive bacteria, and the dye, Brilliant Green which is selective for coliforms. This confirmatory medium (BGB) is not used for the initial or presumptive test because it is apt to be toxic to low numbers of bacteria. The production of gas in the BGB medium within 48 hr at 35 C constitutes a positive confirmed coliform test.

This is normally the extent of the coliform test. If, however, there is some doubt that the organisms present are coliforms, a completed test is required in which bacteria from positive tubes are isolated on Eosin Methylene Blue Agar (EMB) and examined microscopically to confirm that they are gram negative rods, that they are not spore formers, and that the isolates will ferment lactose with the production of gas. For more details concerning this test, the student is referred to Reference 1 and Figure II-B-I.

To enumerate the number of coliforms present, ten-fold dilutions of the water sample are planted in a series of tubes of media. The distribu-

tion of positive tubes among the dilutions can be statistically related to the number of coliforms present in the original sample and is called the Most Probable Number (MPN). Tables that relate the occurrence of positive tubes to MPN appear in Reference 1.

The number of coliform present which are of fecal origin can be estimated by incubating a sample from all positive presumptive lactose tubes in E.C. broth medium at 44.5 $\pm$ 0.5 C for 24 hr (this can be done in parallel with confirmation in Brilliant Green Bile broth). Coliforms of fecal origin are able to grow and produce gas in this medium at this elevated temperature while non-fecal coliforms cannot. Thus, an MPN based upon positive E.C. broth tubes represents the fecal coliforms while an MPN based upon the BGB tubes estimates the total number of coliforms present.

Procedure

A. Drinking Water

1. Collect samples of raw and finished drinking water in sterile, widemouth bottles to which has been added a crystal of sodium thiosulfate to destroy chlorine.
2. To each of 5 tubes of double-strength sterile Lauryl Tryptose Lactose Broth (LTB) containing inverted vials (Durham tubes), add a 10 mℓ water sample. Incubate at 35 C for 24 and 48 hr. If gas appears in any of the vials during the incubation period, the tube is a positive presumptive.
3. Confirm all positive presumptive tubes in Brilliant Green Bile broth (BGB) by transferring a loopful of medium from the former to the latter. Incubate these tubes at 35 C for 24-48 hr. A positive tube will contain gas within 48 hr.

4. Confirm all positive presumptive tubes in E.C. medium in the same manner as Step 3 above and incubate for 24 hr at 44.5 C in a water bath. A positive tube will contain gas within 24 hr.

5. Prepare a Gram stain of the bacteria growing in the confirmed tubes. Although this is not part of the standard MPN method, it will allow you to observe how well these media select for gram negative bacteria.

B. Wastewater

1. The class should be divided into groups so that wastewater from various stages of a waste treatment process can be examined. Samples should represent raw sewage, settled sewage (primary effluent), secondary effluent, disinfected secondary effluent and, if possible, the receiving water downstream from the discharge.

2. Samples (100-200 mℓ) should be taken in sterile, wide-mouth bottles and returned for analysis as soon as possible (within 12 hr).

3. MPN values will be based upon a three-tube in each dilution" test. Ten-fold dilutions should be prepared as described in laboratory exercise No. I-A-1. Raw sewage will usually require dilutions of 10^{-5} through 10^{-8}. Inoculate each of 3 tubes of single-strength LTB broth with 1.0 and 0.1 mℓ of the appropriate dilution (this will require 12 tubes of LTB); for primary effluent use dilutions between 10^{-4} and 10^{-7}; for secondary effluent use dilutions between 10^{-3} and 10^{-6}.

4. Incubate at 35 C for 24-48 hr and record all positive presumptive tubes. Confirm in both BGB and E.C. broth as described in Step A.3 and 4 above.

Data Analysis

1. Record the data in tabular form and determine the MPN for total and fecal coliforms for all the samples using the appropriate tables in Reference 1.
2. Comment on the efficiency with which coliforms and fecal coliforms are removed by various waste treatment processes.
3. Compare your individual results with the entire class results and comment on the variations found in the data.

Apparatus

Sterile sample bottles: 100-200 mℓ, wide mouth, preferably with glass stoppers

Dilution bottles: 99 mℓ, containing sterile buffered dilution water (Reference 1, p. 892)

Water bath: 44.5 C $\pm$ 0.5 C

Test tubes with plugs or caps: 20 x 150 mm containing 10 mℓ media.

Reagents

Lauryl Tryptose Broth (Difco); double-strength, ten tubes per student group.

Lauryl Tryptose Broth (Difco); single-strength, twelve tubes per sample

Brilliant Green Broth (Difco); 12 tubes per sample

E.C. Broth (Difco); 12 tubes per sample

Buffered dilution water (See Exercise I-A-1)

EXPERIMENT II-B-2

INDICATOR ORGANISMS - ENUMERATION OF FECAL STREPTOCOCCI BY THE MOST PROBABLE NUMBER (MPN) METHOD

Purpose

To determine the presence of fecal streptococci in water and wastewater and to enumerate these microorganisms using the most probable number (MPN) method.

References

1. Standard Methods for the Examination of Water and Wastewater, 14th ed. American Public Health Association, p 942, 1976.
2. Geldreich, E.E. "Applying Bacteriological Parameters to Recreational Water Quality," J. American Water Works Association, 63:113-120, 1970.

Introduction

Prior to the development of an effective method for the differentiation of fecal and non-fecal coliforms (the E.C. method), there was considerable interest in the potential use of fecal streptococci as indicators of fecal contamination. The majority of the types of streptococci which originate in the feces of man and warm-blooded animals, are not normally found in soil and plants. However, some strains of streptococci such as S. fecalis var. liquifaciens which is commonly found in insects and an atypical S. fecalis which is frequently found on vegetation are occasionally found in the feces of warm-blooded animals. These two streptococcal strains may be found in water and will be

encountered when performing an analysis for fecal streptococci in water, thus giving results which are of questionable sanitary significance.

Despite these difficulties, analyses for the presence of fecal streptococci are frequently performed in parallel with the coliform test. The ratio of the number of fecal coliforms to the numbers of fecal streptococci in a raw water source may give an indication of the origin of the fecal material. A fecal coliform/fecal streptococci ratio of <1 is indicative of non-human, warm-blooded animal contamination; a ratio of 3-4 is indicative of human fecal contamination and a ratio of 1-3 is indicative of a mixture of animal and human wastes.

The laboratory analysis for the presence of these organisms in water is performed using the presumptive medium, Azide Dextrose Broth, and the confirming medium, Ethyl Violet Azide Broth. Growth present in the latter medium is considered to be a positive test for fecal streptococci.

Procedure

1. Collect samples of wastewater at various stages of treatment (raw sewage, primary effluent, secondary effluent) and receiving water just prior to analysis in 100-200 mℓ sterile, wide-mouth bottles using the method described in Experiment II-B-1 (Coliform Analysis).
2. Inoculate 3 tubes of Azide Dextrose Broth with each dilution of test sample. For raw sewage samples, use ten-fold dilutions from 10^{-5} to 10^{-8}; for primary effluent, use 10^{-4} to 10^{-7} dilutions; for secondary effluent, use 10^{-3} to 10^{-6} dilution; and for receiving waters use dilutions from 10^{-1} to 10^{-4} (see Experiment I-A-1 for dilution method).

3. Incubate the inoculated tubes at 35 C for 24-48 hr. Bacterial growth in the tube is considered a positive presumptive test.
4. From each positive presumptive tube, inoculate a tube of Ethyl Violet Azide Broth by transferring a loopful of growth material from the positive Azide Dextrose tube.
5. Incubate the Ethyl Violet Azide Broth tubes at 35 C for 24-48 hr and record the presence or absence of growth as indicated by turbidity.
6. Gram stain material from the positive Ethyl Violet Broth tubes and note the presence of short-chained streptococci.

Data Analysis

1. Record the number of positive tubes observed both in the presumptive and confirmed tests. Using a standard table, calculate the fecal streptococci MPN per 100 mℓ.
2. Determine the reduction in numbers of streptococci by various waste treatment processes. Compare these reductions to those obtained for coliform MPN and fecal coliform MPN. Are they different? Explain.

Apparatus

Sterile sample bottles, 100-200 mℓ wide-mouth, preferably with glass stoppers.

Dilution bottles, containing 99 mℓ buffered water, one bottle for every two logs of dilution required.

Test tubes, with plugs or caps, 20x150 mm containing 10 mℓ media.

Reagents

Azide Dextrose Broth; (Difco), single strength, 12 tubes per sample.

Ethyl Violet Azide Broth; (Difco), 12 tubes per sample.

Buffered dilution water; (See Exercise I-A-1).

EXPERIMENT II-B-3

ENUMERATION OF INDICATOR ORGANISMS BY THE MEMBRANE FILTER TECHNIQUE

Purpose

To determine the presence of bacterial indicators of water quality using the membrane filter technique.

References

1. Standard Methods for the Examination of Water and Wastewater, 14th ed. American Public Health Association, P. 928, 1976.

Introduction

Application of the membrane filter technique to the enumeration of viable bacteria in a water sample is analogous to the standard technique of plate counting. However, in this instance, a volume of water is filtered through a cellulosic membrane of small porosity (<0.45μm). The bacteria present in the water sample are impinged on the surface of the filter and will grow into colonies when the filter is saturated with an appropriate growth medium. This method allows the technician to determine the presence of bacteria in large volumes of water - volumes that are indeed only limited by the filter clogging properties of the water sample.

Appropriate special media have been devised for the growth and differentiation of coliforms, fecal coliforms and fecal streptococci. The membrane filter (MF) test for coliforms employs M-Endo medium upon which all coliforms will produce red or pink colonies with a golden-greenish metallic sheen. Non-coliforms do not produce such colonies.

Fecal coliforms are grown on a special M-Fecal coliform medium (M-FC). In this method the filter is placed on the medium, enclosed in a small culture dish which is in turn encased in a plastic bag and incubated submerged in a 44.5 C water bath for 24 hr.

Fecal streptococci are incubated on either KF or M-Enterococcus agar media. These selective media contain 2,3,5 triphenyl tetrazolium chloride which, when reduced, colors colonies of fecal streptococci a dark red.

The main advantages of the membrane filter methods are: (1) the results are obtained as a direct bacterial count, (2) the complete test takes only 18-24 hr, and (3) large volumes of water can be examined. The disadvantages are: (1) the filters readily clog with suspended material thus limiting the volume that can be examined and, (2) the presence of extraneous material on the filter will frequently interfere with the resulting growth.

Procedure

1. Examine the following samples: polluted and unpolluted raw water, and finished (treated) drinking water. These samples should be collected in sterile containers. Volumes of 100 mℓ raw water and between 100-1000 mℓ finished water will usually suffice.
2. Set up a membrane filter unit similar to that described in Reference 1, p. 928. Carefully place a sterile, 0.45 μm porosity 47 mm diameter filter on the filter support. Place the filter funnel assembly on top of the membrane filter and clamp to form a tight seal. Attach the filter unit to a vacuum flask and filter.

Filters can be handled aseptically using forceps which have been sterilized by dipping their ends in isopropyl alcohol and igniting. Use when they have cooled.

3. Place at least 100 mℓ sample in the funnel and pull through the filter by vacuum. If the number of bacteria per filter is anticipated to be greater than 50-100, then dilutions should be prepared. For example, 1 mℓ sample can be added to 100 mℓ sterile water and filtered. After the sample has been filtered, rinse the funnel twice with 20 mℓ sterile water and filter. (The instructor will inform you of the need for dilutions).
4. When filtration is complete, aseptically remove the filter and place (filter side up) onto appropriate nutrient medium. Place on medium in such a manner that no air is trapped between the filter and the nutrient surface.
5. Coliform determinations are made using M-Endo medium. Pipette 1.5-2 mℓ M-Endo medium onto a 47 mm bibulous pad which has been aseptically placed in a sterile 47 mm petri dish. Incubate at 35 C for 18-24 hr.
6. Fecal coliform determinations are made using M-Fecal coliform medium. Continue as in Step 5 above except seal the culture dish with masking tape or other suitable tape; place the dishes in a plastic bag; seal tightly and incubate submerged in a 44.5 C water bath for 18-24 hr.
7. Fecal streptococci determinations are made by placing the filter onto the surface of solidified KF agar. This agar has been prepared and placed in standard petri dishes (about 15 mℓ per dish) and allowed to solidity. Incubate at 35 C for 24-48 hr.

Results

1. Count the typical colonies produced on each medium. Compare the number of fecal and total coliforms and determine the ratio of fecal coliforms to fecal streptococci.
2. What relationship exists (if any) between total and fecal coliforms and fecal streptococci determined by MF and MPN techniques?

Apparatus

Sterile membrane filters: 47 mm diameter, 0.45 µm pore size, Millipore Filter Corp. or equivalent; 9 per student group.

Absorbent Pads: 47 mm diameter, Millipore Filter Corp. or equivalent; 9 per student group.

Membrane Filter Unit (funnel, base, clamp, filter flask and hose); Millipore Filter Corp. or equivalent.

Forceps.

Pipettes; sterile, 1 mℓ and 5 mℓ.

Reagents and Media

250 mℓ portions of sterile distilled water in 500 mℓ erlenmeyer flasks; 2 portions per student group.

M-Endo Broth (Difco); 10 mℓ per student group.

M-Fecal Coliform Broth (Difco); 10 mℓ per student group.

KF Streptococcal agar (BBL); standard petri dishes containing 15 mℓ of agar per dish; 3 per student group.

III. MICROBIOLOGY OF WATER AND WASTEWATER TREATMENT

EXPERIMENT III-A-1

THE MICROBIAL COMMUNITY IN ACTIVATED SLUDGE

Purpose

The purpose of this experiment is to demonstrate the development of the microbial community of activated sludge from the microorganisms of settled sewage and to examine the ability of this developing culture to flocculate and settle as a function of time of development. A second objective is to observe the effect of elevated organic carbon concentration on these two characteristics of this culture.

References

1. Pipes, W.O. "Bulking of Activated Sludge." Advances in Applied Microbiology, 9:185-234, 1967.
2. Standard Methods for the Examination of Water and Wastewater, 14th ed. American Public Health Association, pp. 127, 129, 130, 132, 532, 550, 1976.
3. Pike, E.B. and C.R. Curds. "The Microbial Ecology of the Activated Sludge Process," in Microbial Aspects of Pollution, Sykes, G. and Skinner, E.A. eds., Academic Press, New York, 1971.

Introduction

The success of the activated sludge process depends on rapid nutrient removal and is related to the ability of bacteria and other microorganisms to cling together in large flocculant particles which can be removed from the effluent stream by settling. A portion of the settled microbiological floc or sludge is then recirculated to seed the incoming waste.

One of the factors that influence the settling properties of the biomass is the growth rate of the microorganisms or the so-called "sludge age."

The microbiological community responsible for nutrient removal is collectively referred to as activated sludge. The development of the optimum community capable of the most efficient nutrient removal is a time-dependent microbial succession. By definition, in a succession, each characteristic group is dependent upon the environmental conditions established by the preceding group. Conditions are therefore selective for the particular group which follows. Consequently, under a given set of parameters such as dissolved oxygen concentration, pH, temperature, organic and inorganic nutrient concentrations, and cell residence time, selective conditions develop so that a microbial succession is directional, repeatable and predictable. In an aqueous system, this time dependent process can be illustrated, in principle, by Figure III-A-I.

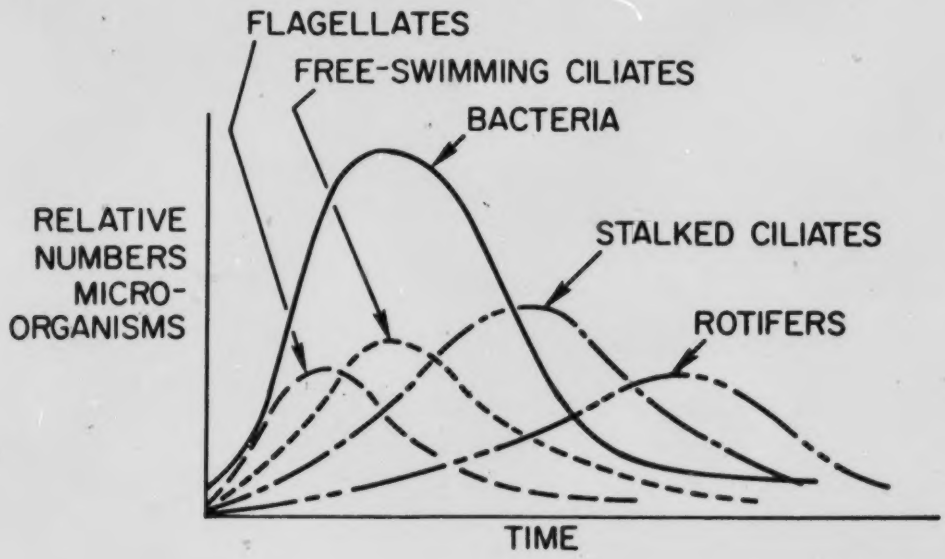


FIGURE III-A-I. SELECTION OF MICROBIAL TYPES IN ACTIVATED SLUDGES

Bacteria predominate not only in numbers, but are are the single most important group of microorganisms responsible for nutrient removal. In addition, they also play a significant role in the production of extracellular materials that are necessary for successful flocculation.

The different groups of protozoa and rotifers have a smaller impact on soluble nutrient removal, but are important members of the activated sludge community. They are thought to be "polishers" and to increase the effluent quality mainly by the removal of suspended solids. Protozoa are highly effective bacterial predators and may graze on the floc material.

In addition to nutrient removal, the success of the activated sludge treatment process is also dependent upon flocculation of the particulate material for effective settling and consequent separation of the solids from the effluent. Production of the bacterial extracellular material required for flocculation is also a time dependent process and it appears that the extracellular material production generally takes place in old cells.

One of the often encountered problems in activated sludge plants is poor settling sludge which leads to poor effluent quality. Different types of settling problems include: dispersed growth - no floc formation; rising sludge - sludge carried to the surface by nitrogen gas produced by denitrification in the sedimentation basin; and bulking sludge - slow settling sludge with very poor compaction characteristics.

The microbiological community of bulking sludge very often consists of an unusually high concentration of filamentous microorganisms. Both filamentous bacteria such as Sphaerotilus or Beggiatoa as well as fungi

have been implicated in activated sludge bulking. The fact that such a large and uncharacteristic proportion of these organisms are present indicates that different selective conditions exist and that it is an improper environment for the development of a flocculent activated sludge community. Some of the conditions thought to encourage bulking are: (i) low dissolved oxygen concentrations; (ii) Low concentrations of the inorganic nutrients nitrogen and phosphorus; (iii) low pH (less than 6); (iv) high concentrations of soluble carbohydrates creating a large C:N ratio. Many of these conditions are characteristically selective for fungi and filamentous bacteria.

Procedure

1. This experiment will be performed as a class exercise with each student being responsible for the examination of a culture of a particular "age" or period of aeration.
2. The following procedure should be performed starting 15 days prior to the date of the experiment:
   a. Set up ten 2ℓ beakers marked at 1.5 ℓ. Insert a Pasteur pipette and connect it to a source of filtered compressed air.
   b. Using the following schedule start up cultures from domestic settled sewage:

Culture No.	Set Up Time, Days Prior to Class Period
1	15
2	10
3	7
4	5
5	4
6	3
7	2
8	1
9	7 (with 1.0 g glucose
10	7 (with 2.0 g glucose

3. To start cultures 1-8 use the following procedure:

   a. Add 10 mℓ Metrecal and 5 mℓ settled sewage ot the beakers.

   b. Add tap water up to the 1.5 ℓ mark.

   c. Commence aeration.

4. Cultures 9 and 10 are developed from settled sewage, Metrecal and glucose as follows:

   a. To beaker 9, add 10 mℓ Metrecal, 1.0 g glucose and 5 mℓ settled sewage.

   b. To beaker 10, add 10 mℓ Metrecal, 2.0 g glucose and 5 mℓ settled sewage.

   c. Add tap water to the 1.5ℓ mark in both beakers.

   d. Commence aeration.

5. Aerate thoroughly, but gently. Add distilled water periodically to maintain liquid volume at 1.5ℓ.

6. Scrape down sides of beakers daily to keep bacteria in suspension. Be careful to prevent carry-over of solids from one beaker to the next during this procedure.

7. On the day of the experiment mix the beaker contents and gently pour into 1ℓ graduate cylinders. Perform the following observations on each culture:

   a. Perform a sludge volume index (SVI) measurement and a settling curve measurement (note the level of the sludge blanket at several time intervals over a 1 hr period).

   b. Make macroscopic observations of each sample regarding:

      (i) setting ability

      (ii) supernatant clarity

      (iii) floc size

c. Examine immediately another aliquot of the supernatants under the phase contrast microscope (if available) using a Petroff-Hauser counting chamber. Determine relative numbers of each of the following groups of microorganisms:

(i) bacteria

(ii) flagellated protozoa

(iii) free swimming ciliates

(iv) stalked ciliates

(v) rotifers and other higher animals

(vi) fungi and filamentous bacteria (indicate which type observed).

d. From the liquid remaining in the beaker, remove a sample of the flocculant material and make 1.c. observations using a Petroff-Hauser counting chamber. Determine the relative numbers of: (i) bacteria, (ii) flagellated protozoa, (iii) free swimming ciliates, (iv) stalked ciliates, (v) rotifers and other higher animals and (vi) fungi and filamentous bacteria.

8. If desired, COD or organic carbon determinations and turbidity measurements can be made on the SVI supernatant. Suspended solids and volatile suspended solids can be made of the material remaining in the beakers (See Reference 2).

Data Analysis

1. Tabulate SVI values and rate the observations from 1 (good) to 5 (poor):

a. settling ability

b. supernatant clarity

c. floc size

Briefly describe the characteristics in 1.a. through 1.c. for each sample.

2. Tabulate the l.c. observations indicating relative numbers of:

   a. bacteria

   b. flagellated protozoa

   c. free swimming ciliates

   d. stalked ciliates

   e. rotifers and other higher animals

   f. fungi and filamentous bacteria, in the supernatant and the mixed culture

   g. microscopic floc characteristics

3. To start an activated sludge plant with no available activated sludge as a starter, suggest two procedures which might be used to develop a well settling sludge.

4. In what way did the macroscopic and microscopic observations vary with culture or sludge age? Explain.

5. Optional: tabulate "physical/chemical" data collected for all samples in Step 8 and discuss the differences found in the various samples.

Apparatus

Beakers, 2ℓ: 10 per student group.

Compressed air supply with glass wool filter.

Graduate cylinders, 1ℓ: 10 per student group.

Rubber police persons

Pasteur pipettes

Depression slides or Petroff-Hauser counting chambers.

Cover slips

Compound microscope

Phase contrast micorcope

Stopwatch

Optional:

Hotplate and COD apparatus

Turbidity meter

Membrane filter apparatus to hold 47 mm filters

Glass fiber filters: Whatman GF/C, 47 mm diameter

Total Organic Carbon Analyzer: Beckman 900 or equivalent

Reagents

Primary settled domestic sewage - starter culture

Metrecal or equivalent commercial preparation with C/N ratio of approximately 5:1

Glucose

COD Reagents: See Reference 2, p. 550

Organic Carbon Standard: dissolve 5.571 g anhydrous sodium oxalate $Na_2C_2O_4$, in 1ℓ distilled water 1 mℓ ≡ 1.0 mg C

KINETIC MODELS FOR THE CONTINUOUS CULTURE OF MICROORGANISMS

Introduction

Two experiments are presented in this section which are designed to illustrate: (i) one of the kinetic models used for activated sludge to relate growth rate to growth-limiting substrate concentration and (ii) the use of continuous flow laboratory activated sludge units for determining the growth constants of yield and endogenous decay rate and the relationship between growth rate and soluble substrate concentration as measured by soluble COD - a collective substrate parameter.

The activated sludge process is a biological treatment scheme for wastewaters in which a mixed and variable enrichment culture of microorganisms grow at the expense of organic and inorganic substrates present in wastewater. The process operates according to the flow scheme in Figure III-B-I. From a microbiological point of view it is important to note that the flow sheet includes a recycle sludge (or microorganism stream) so that the net growth rate of the sludge is made largely independent of the hydraulic dilution rate and largely dependent on the separate operation of sludge wasting. It is also important when considering the types of microorganisms that may develop in activated sludge to realize that the sludge separation device most commonly utilized is a gravity separator. Thus, microorganisms that settle readily (i.e. flocculent growths) will tend to be enriched over those that do not settle well. Because of the basic underlying microbial nature of the process some considerable success has been obtained in modelling and predicting the performance of activated sludge processes using the

kinetics of microbial growth. The following development illustrates how such models are applied to the activated sludge process.

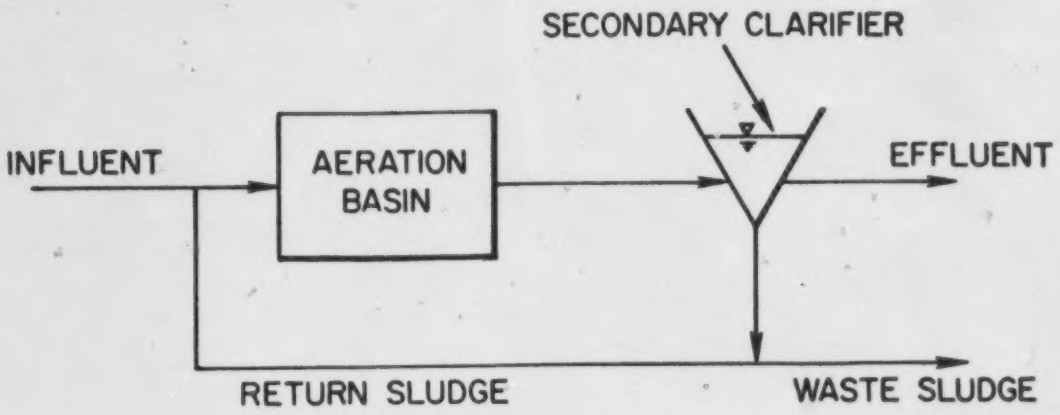


FIGURE III-B-I. ACTIVATED SLUDGE PROCESS FLOW SHEET

When the rate of many enzyme reactions are plotted against their initial substrate concentrations a rectangular hyperbolic curve is often obtained (Figure III-B-II).

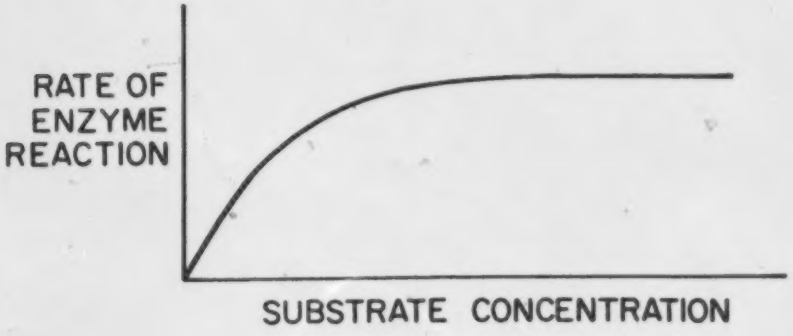


FIGURE III-B-II. RATE OF ENZYME REACTION AS A FUNCTION OF SUBSTRATE CONCENTRATION

In attempting to account for the plateau in this curve, Michaelis and Menten (Reference 1) postulated the existence of an enzyme substrate comple, (ES) and the two types of reactions given below:

$$E + S \underset{K_2}{\overset{K_1}{\rightleftharpoons}} ES$$

$$ES \xrightarrow{K_3} E + P$$

where

E = enzyme concentration

S = substrate concentration

P = product concentration

The controlling factor at high substrate concentration is the concentration of the ES complex. Therefore, the curve flattens because there is more substrate present than the enzyme can combine with. When the substrate concentration is low, the rate of an enzyme action parallels the substrate concentration if all other variables are kept constant.

Based upon the work of Michaelis and Menten the following equation can be derived:

$$V = \frac{V_m \cdot S}{K_m + S}$$

where

V = reaction rate

V_m = the maximum reaction rate

S = substrate concentration

K_m = Michaelis-Menten constant,

= substrate concentration at half maximum reaction rate.

This equation has been used with collective parameters to represent microorganism and growth-limiting substrate concentration for heterotrophic microorganisms, to describe the mass growth rate of activated sludge in the following form:

$$\mu = \frac{\hat{\mu}\, s}{K_s + s}$$

where

μ = growth rate = $\frac{\text{mg VSS}}{\text{mg VSS in system, day}}$

$\hat{\mu}$ = maximum growth rate, day^{-1}

s = substrate concentration = mg BOD/ℓ or mg dissolved COD/ℓ.

K_s = substrate concentration at half maximum growth rate

VSS = collective parameter for microorganism mass, volatile suspended solids, mg/ℓ

COD = collective parameter for growth-limiting substrate, chemical oxygen demand, mg/ℓ

BOD = collective parameter for growth-limiting substrate, 5 day, 20 C biochemical oxygen demand, mg/ℓ

In kinetically describing activated sludge systems it is useful to relate the growth-rate to the rate of substrate removal which is of significance to the performance of the process. This can be state as follows:

$$m = Yq$$

where

Y = yield = $\frac{\text{mg VSS formed}}{\text{mg COD removed}}$

q = substrate removal rate = $\frac{\text{mg COD removed}}{\text{mg VSS in system, day}}$

$$= \frac{F(s_o - s_1)}{V X_1} = \frac{s_o - s_1}{X_1 \theta}$$

where

F = feed flow rate

s_o = influent substrate concentration

s_1 = effluent substrate concentration

V = reactor volume

χ = cell concentration

θ = hydraulic residence time = V/F

In activated sludge systems and other biological waste treatment processes where growth rates are very low, it is important to consider the effect of microorganism decay rates when assessing the net growth rate of a system. The net growth rate, or cell dilution rate can be expressed as:

$$D_c = m - k_d$$

where

k_d = microbial mass decay rate, day^{-1}

or

$$D_c = Y\,q - k_d$$

The characterization of a wastewater for treatment by the activated sludge process can be achieved by performing several experiments at different values of D_c or q. Since D_c and q can both be derived from measured experimental values, the kinetic "constants" Y and k_d can be determined from a plot of D_c and q as in Figure III-B-III.

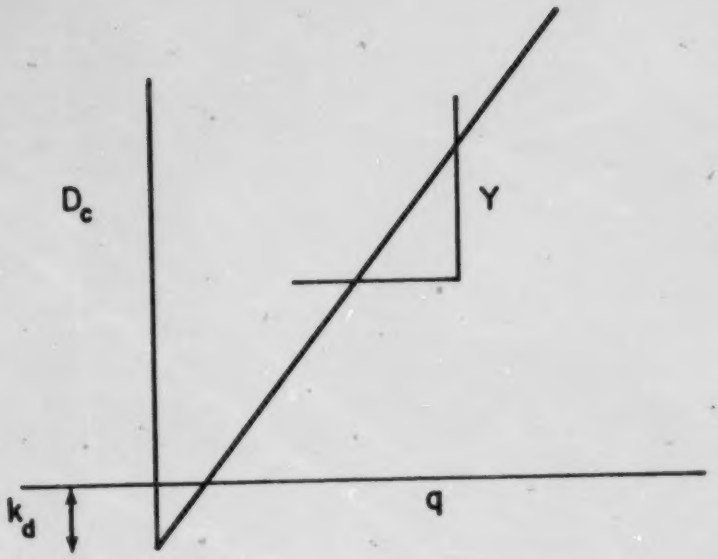


FIGURE III-B-III. DETERMINATION OF YIELD AND ENDOGENOUS DECAY CONSTANTS

In addition, measurement of q and s_1, and a knowledge of the value of Y will allow determination of the kinetic constants $\hat{\mu}$ and K_s.

Since,

$$\mu = \frac{\hat{\mu}\, s_1}{K_s + s_1} \quad \text{and} \quad \mu = Yq$$

Therefore

$$q = \frac{1}{Y} \frac{\hat{\mu}\, s_1}{K_s + s_1}$$

This equation can be linearized in several ways to determine $\hat{\mu}$ and Ks. It should be noted that at high substrate concentrations, i.e., $s_1 >> K_s$, the relationship between q and s_1 becomes $q = \hat{\mu}/K_s$ = constant, while at low substrate concentrations, i.e. $K_s >> s_1$, $q = (\hat{\mu}/Y\, K_s)\, s_1 = n\, s_1$, i.e. there is a linear (pseudo first-order) relationship between q and s_1 where the constant of proportionality n is equal to $\hat{\mu}/Y$.

EXPERIMENT III-B-1A

DETERMINATION OF THE MICHAELIS-MENTEN CONSTANT AND MAXIMUM REACTION RATE OF THE ENZYME UREASE

Purpose

To determine the Michaelis-Menten constant and the maximum reaction rate of the enzyme urease.

References

1. Standard Methods for the Examination of Water and Wastewater, 14th ed. American Public Health Association, p. 412, 1976.
2. Fruton, J.S. and Simmonds, S. General Biochemistry, 2nd ed. J. Wiley and Sons, Inc.

Procedure

1. By diluting the appropriate urea solution (either 1% or 6%) prepare a duplicate series of test tubes (total of 20) containing exactly 5 mℓ of the urea solutions shown in Table III-B-I.

TABLE III-B-I. STRENGTH OF UREA SOLUTIONS

Tube No.	Urea Concentration		Required Dilutions
	M	% (w/v)	
1	0.000	0.00	5 mℓ Distilled water
2	0.016	0.10	2 mℓ 1%* + 18 mℓ water
3	0.040	0.25	5 mℓ 1%* + 15 mℓ water
4	0.080	0.50	10 mℓ 1%* + 10 mℓ water
5	0.120	0.75	15 mℓ 1%* + 5 mℓ water
6	0.160	1.00	5 mℓ 1%*
7	0.250	1.50	3 mℓ 6%* + 9 mℓ water
8	0.333	2.00	4 mℓ 6%* + 8 mℓ water
9	0.666	4.00	8 mℓ 6%* + 4 mℓ water
10	1.000	6.00	5 mℓ 6%

*Urea solution strength

Place all the tubes, into a 37 C constant temperature water bath.

2. Dissolve 6.0 g K_2HPO_4 and 4.0 g KH_2PO_4 in about 60 mℓ double distilled water in a small beaker. Add 2.0 g urease, stir gently to dissolve, transfer to a 100 mℓ volumetric flask, dilute to volume and mix. Place this flask in the 37 C water bath.

3. After the solutions have reached thermal equilibrium add exactly 1 mℓ of the enzyme solution to both of the No. 1 tubes. Record the time and mix the contents gently by swirling. Using the same technique add 1 mℓ enzyme solution to each successive tube at a constant interval e.g. 20-25 sec.

4. Beginning exactly 10 min after the first addition of enzyme add 2 mℓ 1 N HCl to each tube in the same sequence and time intervals as before so that in all cases the enzyme is inhibited after exactly 10 min of action.

5. Remove all tubes from the water bath and determine the ammonia produced in each tube by an appropriate dilution with subsequent nesserlization as follows:

   a. Dilute 1.0 mℓ solution to about 80 mℓ with distilled water in 100 mℓ volumetric flask.

   b. Add 5 mℓ Nessler reagent while swirling the flask vigorously.

   c. Add water to volume and mix the contents of the flask.

   d. Measure the optical density at 490 nm after 10 min using the zero concentration tube as a blank.

6. Prepare a standard curve for the ammonia determination as follows:

   a. Add 3.0 mℓ $(NH_4)_2SO_4$ solution (1.322 mg/mℓ) to 27.0 mℓ distilled water.

   b. Mix and nesslerize in duplicate 1, 2, 3 and 4 mℓ of the solution prepared in Step 6a using the technique given in Step 5 above.

EXPERIMENT III-B-1A

DETERMINATION OF THE MICHAELIS-MENTEN CONSTANT AND MAXIMUM REACTION RATE OF THE ENZYME UREASE

Purpose

To determine the Michaelis-Menten constant and the maximum reaction rate of the enzyme urease.

References

1. Standard Methods for the Examination of Water and Wastewater, 14th ed. American Public Health Association, p. 412, 1976.

2. Fruton, J.S. and Simmonds, S. General Biochemistry, 2nd ed. J. Wiley and Sons, Inc.

Procedure

1. By diluting the appropriate urea solution (either 1% or 6%) prepare a duplicate series of test tubes (total of 20) containing exactly 5 mℓ of the urea solutions shown in Table III-B-I.

TABLE III-B-I. STRENGTH OF UREA SOLUTIONS

Tube No.	Urea Concentration		Required Dilutions
	M	% (w/v)	
1	0.000	0.00	5 mℓ Distilled water
2	0.016	0.10	2 mℓ 1%* + 18 mℓ water
3	0.040	0.25	5 mℓ 1%* + 15 mℓ water
4	0.080	0.50	10 mℓ 1%* + 10 mℓ water
5	0.120	0.75	15 mℓ 1%* + 5 mℓ water
6	0.160	1.00	5 mℓ 1%*
7	0.250	1.50	3 mℓ 6%* + 9 mℓ water
8	0.333	2.00	4 mℓ 6%* + 8 mℓ water
9	0.666	4.00	8 mℓ 6%* + 4 mℓ water
10	1.000	6.00	5 mℓ 6%

*Urea solution strength

Place all the tubes, into a 37 C constant temperature water bath.

2. Dissolve 6.0 g K_2HPO_4 and 4.0 g KH_2PO_4 in about 60 mℓ double distilled water in a small beaker. Add 2.0 g urease, stir gently to dissolve, transfer to a 100 mℓ volumetric flask, dilute to volume and mix. Place this flask in the 37 C water bath.

3. After the solutions have reached thermal equilibrium add exactly 1 mℓ of the enzyme solution to both of the No. 1 tubes. Record the time and mix the contents gently by swirling. Using the same technique add 1 mℓ enzyme solution to each successive tube at a constant interval e.g. 20-25 sec.

4. Beginning exactly 10 min after the first addition of enzyme add 2 mℓ 1 N HCl to each tube in the same sequence and time intervals as before so that in all cases the enzyme is inhibited after exactly 10 min of action.

5. Remove all tubes from the water bath and determine the ammonia produced in each tube by an appropriate dilution with subsequent nesserlization as follows:

   a. Dilute 1.0 mℓ solution to about 80 mℓ with distilled water in 100 mℓ volumetric flask.

   b. Add 5 mℓ Nessler reagent while swirling the flask vigorously.

   c. Add water to volume and mix the contents of the flask.

   d. Measure the optical density at 490 nm after 10 min using the zero concentration tube as a blank.

6. Prepare a standard curve for the ammonia determination as follows:

   a. Add 3.0 mℓ $(NH_4)_2SO_4$ solution (1.322 mg/mℓ) to 27.0 mℓ distilled water.

   b. Mix and nesslerize in duplicate 1, 2, 3 and 4 mℓ of the solution prepared in Step 6a using the technique given in Step 5 above.

Data Analysis

1. Present data in tabular format and plot the following curves:

   a. Initial reaction rate, V, vs initial substrate concentration, S.

   b. S/V vs S.

   c. 1/V vs 1/S.

2. From these curves determine the Michaelis-Menten Constant (K_m) and the maximum reaction rate ($\hat{\mu}$) for urease. How do your values compare with those reported in the literature?

Apparatus

Water bath, to hold 20 test tubes at 37 C

Spectrophotometer to read at 420 nm.

Test tubes: 48 per student group.

Volumetric flasks, 100 mℓ: 20 per student group.

Reagents

Urea solution, 1% w/v.

Urea solution, 6% w/v.

Urease solution, 2 g urease/100 mℓ distilled water.

K_2HPO_4, reagent grade: 6 g per student group.

K_2PO_4, reagent grade: 4 g per student group.

HCl solution, 1N.

$(NH_4)_2SO_4$ standard solution, 1.322 mg/mℓ.

Nessler reagent (Reference 1, p. 412).

Double-glass-distilled water.

EXPERIMENT III-B-1B

DETERMINATION OF KINETIC CONSTANTS FOR ACTIVATED SLUDGE

Purpose

The purpose of this experiment is to determine the mass yield and endogenous decay coefficients and the relationship between activated sludge growth rate and substrate concentration expressed as soluble COD utilizing continuous flow activated sludge units.

References

1. Jenkins, D. and Garrison, W.J. Water Pollution Control Fed. 40: 1905, 1968.
2. Pearson, E.A. Kinetics of Biological Treatment. Lecture Series on Advances in Water Quality Improvement, University of Texas, Austin, Texas, 1966.
3. Standard Methods for the Examination of Water and Wastewater, 14th ed. American Public Health Association, 1976.

Procedure

1. This experiment entails the operation of three laboratory-scale continous flow activated sludge plants at steady state on a synthetic sewage feed for a period of 2 weeks. These units will have been set in operation at substrate removal rates of approximately 0.1, 0.6 and 3.0 mg COD removed/mg VSS-day. A typical aeration and sedimentation design is presented in Figure III-B-IV and the flow diagram is shown in Figure III-B-V. Details of construction are

given in the Apparatus Section of this experiment. The student groups will be responsible for the daily operation of the activated sludge units including housekeeping, sampling, flow measurements, analyses and preparation of feed.

2. Preparation of Feed: The feed is a "synthetic sewage" whose major components are egg albumin, beef extract and K_2HPO_4 as shown in Table III-B-II. This feed is "soluble" and has as its principal carbon and energy source the high molecular weight egg albumin. Beef extract is added as a further carbon and energy source and to provide a source of growth factors. These two components are combined in a COD ratio of approximately 3:1.

TABLE III-B-II. SUBSTRATE COMPOSITION FOR ACTIVATED SLUDGE EXPERIMENT

Constituent	Quantity	Concentration mg/ℓ	COD mg/ℓ
Egg albumin (Difco)	5.12 g	284	300
Beef extract (Difco)	1.62 g	89.6	62
K_2HPO_4	0.94 g	52.2	
Ethanolic phenolphthalein solution	1.0 mℓ		35
Total COD			397
NaOH (4N)	10.0 mℓ		
Tap water	18.0 ℓ		

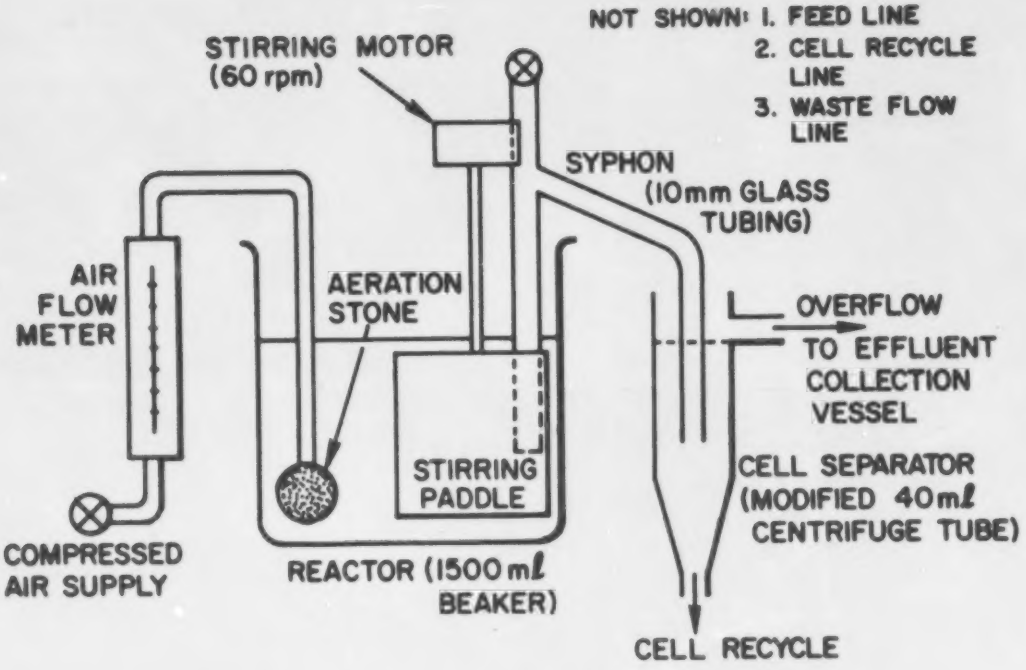


FIGURE III-B-IV. PICTORIAL DIAGRAM OF LABORATORY ACTIVATED SLUDGE UNIT

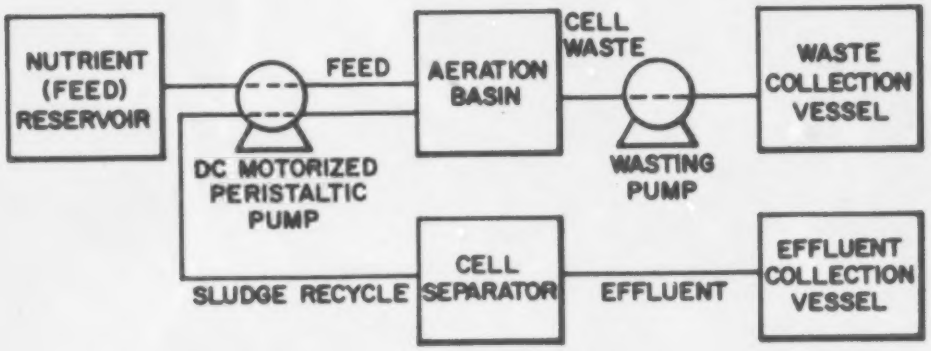


FIGURE III-B-V. BLOCK FLOW DIAGRAM OF LABORATORY ACTIVATED SLUDGE UNIT

The addition of phenolphthalein is to provide a visual indicator of gross bacterial contamination of the feed which is indicated by a pH drop and consequent phenolphthalein discoloration. The adjustment of the feed pH to 10.5 is an additional precaution against gross bacterial contamination of the feed and has no effect on the pH of the activated sludge reactor which stays within the range of 7.2 - 7.5.

3. Housekeeping of Activated Sludge Units: The cleaning schedule for the aeration and sedimentation basins must be followed rigorously:

   a. Daily, clean interior walls and splash zone above aeration basin liquid level with a bristle brush. Clean sedimentation basin in same fashion using a smaller brush. Check that sludge return line from sedimentation basin is open. To be certain, rod out with a piece of wire. Clean all feed and return lines by reaming between thumb and fingers after sampling. Clean collection bottles thoroughly. Check water level in water scrubber on air supply.

   b. Weekly, change and clean feed bottle.

4. Sampling and Flow Measurements: The following samples should be taken once per day for each unit:

   a. Mixed liquor sample for the following measurements: mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS) and sludge volume index (SVI). Take two 10 mℓ grab samples by wide-tip pipette from center of aeration basin 1 hr after cleaning the basin.

   b. Effluent sample for soluble COD measurement: take a 50 mℓ grab sample from sedimentation basin overflow 1 hr after cleaning aeration basin.

c. Effluent sample for suspended solids and volatile suspended solids measurement: measure total effluent volume from a day's operation, mix well; take aliquot (approximately 25-200 mℓ) whose size will depend on SS content of effluent. Filter such a volume as will produce a reasonable amount (2-5 mg) of solids on the filter.

d. Waste sludge sample for SS or VSS measurement: measure total waste sludge volume from a day's operation; mix well and take 10 mℓ aliquot.

e. Influent feed sample for COD determination (50 mℓ):

NOTE: The volumes of all samples taken from the aeration basin must be accounted for in sludge wasting computations.

The following samples should be taken <u>once per week for each unit</u>:

a. Effluent sample for determination of nitrogen forms: take a 300 mℓ grab sample from sedimentation basin overflow.

b. Influent sample for determination of nitrogen forms (200 mℓ).

5. <u>Analyses and Measurements</u>: The analytical techniques to be used are appended in outline form at the end of this experiment. In general, techniques from Reference 3 should be used.

The following analyses and measurements should be performed <u>once per day</u>:

a. SS on aeration basin contents, effluent and waste sludge

b. VSS on aeration basin contents, effluent and waste sludge

c. SVI on aeration basin contents

d. Soluble COD on effluent grab sample

e. Total COD on influent feed

f. Daily effluent volume

g. Daily waste sludge volume (include volumes taken from aeration basin during sampling)

h. Check pumping rates for feed, sludge recycle and sludge wasting

The following measurements should be performed once per week on influent and effluent samples: Nitrogen forms (Total oxidized nitrogen, NH_3 and Organic N).

6. Maintenance of Desired Substrate Removal Rate: Since substrate removal rate and net growth rate are related, it is possible to manipulate one of these parameters by changing the other. In this experiment, 3 desired substrate removal rates have been established with a fixed feed COD concentration and a desired flow rate. In order to maintain substrate removal rate, therefore, the only variable that can be manipulated is the aeration basin suspended solids concentration X_1 (recall $q = F(s_o - s_1)/V\ X_1$). This is achieved by variation of the sludge wasting rate. Since sludge wasting may take place both "intentionally" (by the sludge wasting pump) and "unintentionally" (as SS in the secondary effluent) it is necessary to perform the entire daily set of analyses and measurements (COD, SS and flow measurements) before the changes are made in the sludge wasting rate. Changes in sludge wasting rate should be made rather cautiously with the objective of making the desired change gradually over a few days rather than all at once. Target operational parameters for the substrate removal rates 0.2, 0.6 and 3.0 mg COD removed/mg VSS, day and influent flow rates of 3, 3 and 5ℓ/day respectively, are given in the instructions for start-up of the activated sludge units in the Apparatus Section.

Data Analysis

1. Present a concise tabular summary of the data that includes all measured daily parameters and computed daily values of substrate removal rate and net growth rate.
2. Summarize the daily data for substrate removal rate and net growth rate for each unit in graphical format with time. Select periods of data that represent steady state (establish and state your own criteria for steady state).
3. Present a plot of substrate removal rate vs. net growth rate. From this plot determine the line of best fit and derive values for cell yield from the slope D_c/q and for the mass decay rate (negative intercept on D_c axis).
4. Present a plot of substrate removal rate vs. effluent soluble substrate (soluble COD) concentration. What form of relationship appears to fit these data points? Express the line fit for these points as a mathematical statement and determine the value of any constants you may have in your equation.
5. What effect does substrate removal rate have on the settleability of the sludge? What explanations have been offered in the literature to account for these effects?
6. Discuss the effect of sludge growth rate on nitrogen dissimilation. If the activated sludge is known to contain 15% N on a dry VSS basis what fraction of the influent nitrogen is used for sludge synthesis at each substrate removal rate?
7. Assuming the above figure for sludge nitrogen content, can a materials balance for nitrogen be made at each substrate removal rate? If not, why not?

Apparatus

Aeration basin: 1500 mℓ Griffen Beaker placed on adjustable lab-jack.

Transfer to sedimentation basin: Use either a siphon (See Figure III-B-IV) or a beaker with a side arm at the 1000 mℓ mark.

Sedimentation basin: 1-1/2 in i.d. glass tubing drawn to a conical bottom and with side arm attached for overflow. Total volume of about 100 mℓ; upflow zone of about 40 mℓ. (See Figure III-B-IV).

Aeration basin mixing: 2 in x 3 in stirring paddles driven at 60 rpm by synchronous motor (Cramer Model 117 or equivalent).

Aeration basin air supply: Compressed air at 0.2-0.4 ft^3/hr through porous stone diffusers (aquarium type). Pass the air through a glass wool filter (oil trap). A washing bottle containing distilled water and through rotameters. (Brooks-Mite Model 1-38B or equivalent).

Pumps: Use peristaltic pumps with Tygon tubing. Use same pump for feed and sludge recycle pumping and vary the sludge recycle ratio by varying the size of tubing. Suitable pumps are:

Feed/Recycle: Sigmamotor Model T-6 driven by Bodine NSH-54RL Motor and Minarik SH-53 Speed Controller.

Waste sludge: Sigmamotor Model T-8 driven by Bodine NSH-54 RL Motor and Minarik SH-53 speed controller actuated by an interval timer on an intermittent (e.g. 1/hr basis).

Waste sludge is pumped directly from the aeration basin.

Feed container: 5 gal carboy graduated in one liter intervals.

Effluent receiver: 2 gal bottle.

Waste sludge receiver: 1 gal bottle.

Solutions

Synthetic sewage feed:

a. Add 16ℓ hot tap water to a 20ℓ (5 gal) reservoir that has been autoclaved together with its delivery tubing at 121 C for 15 min.

b. Dissolve 5.12 g egg albumin (Difco) in NaOH solution (10 mℓ 4N NaOH in 500 mℓ distilled water) and heat slowly to 80 C and allow to cool. Add to hot water in feed reservoir.

c. Dissove 16.2 g beef extract in 100 mℓ distilled water and autoclave at 121 C for 15 min. Reconstitute volume to 100 mℓ with sterile distilled water. Cool and add 10 mℓ of this solution to the hot water in feed reservoir.

d. Dissolve 93.96 g K_2HPO_4 in 100 mℓ distilled water and autoclave at 121 C for 15 min. Reconstitute volume to 100 mℓ with sterile distilled water. Cool and add 1 mℓ of this solution to the hot water in the feed reservoir.

e. Add 1.0 mℓ 0.5% phenolphthalein in 95% ethanol.

f. Add 10 mℓ 4 N NaOH.

g. Mix contents of feed reservoir and add hot tap water to a volume of 18 ℓ. The pH should be about 10.5 and the feed should appear pink.

Activated sludge: Obtain activated sludge from a sewage treatment plant aeration basin and use to start up laboratory activated sludge units. It may be useful to maintain a laboratory fill-and-draw activated sludge unit (23 hr aeration and 1 hr sedimentation) on synthetic feed prior to the start up of the continuous laboratory activated sludge units.

Activated sludge unit start-up: The following conditions (Table III-B-III) should be used for the three laboratory activated sludge units to

attain approximate substrate removal rates of 0.2, 0.6 and 3.0 mg COD removed/mg VSS-day. Waste sludge volumes will have to be adjusted to account for loss of suspended solids in the effluents. The activated sludge units should be run continuously at steady state for at least two cell residence times prior to the start of the experiments.

TABLE III-B-III. OPERATING CONDITIONS FOR ACTIVATED SLUDGE UNITS

Desired q (mg COD Removed/mg VSS,day)	Reactor Volume ℓ	Influent Flowrate ℓ/day	Feed COD mg/ℓ	Estimated Effluent COD mg/ℓ	Estimated MLVSS mg/ℓ	Target* Was Rate From Aeration Basin	
						mg/ℓ	g/da
0.2	1	3	400	50	5000	12	0.06
0.6	1	3	400	50	2300	200	0.46
3	1	5	400	70	550	1300	0.71

* Maximum value - does not account for SS lost in effluent which should be estimated and subtracted from these values.

Analytical Methods

1. Suspended Solids (SS)
   a. Dry Whatman grade GF/C glass fiber filter in 105 C oven, 30 min.
   b. Cool in desiccator.
   c. Weigh filter to 0.1 mg.
   d. Filter measured sample volume using Millipore filtration apparatus.

e. Dry at 105 C for 1/2 hr.

f. Cool in desiccator and reweigh.

2. Total COD

a. Wear safety glasses.

b. Add 50 mg $HgSO_4$ with scoop to 250 mℓ reflux flask.

c. Pipette in 5 mℓ 0.1N $K_2Cr_2O_7$ solution.

d. Pipette in sample (10 mℓ or aliquot made up to 10 mℓ with distilled water).

e. Add 15 mℓ conc. H_2SO_4 containing Ag_2SO_3 slowly with mixing.

f. Reflux 1 hr on hotplate (Turn condenser water on).

g. Cool, add 6 mℓ distilled water down the condenser.

h. Add 2 drops o-phenanthroline indicator.

i. Titrate with 0.055 N $Fe(NH_4)_2\ SO_4$, to first red color.

j. Run a distilled water blank through whole procedure.

k. Standardize $Fe(NH_4)_2SO_4$ daily against a standard 0.1N $K_2Cr_2O_7$ solution.

3. Soluble COD

a. Proceed as in Step 2 but use the filtrate from the SS test.

4. Sludge Volume Index

a. Take 10 mℓ grab sample from aeration basin.

b. Settle for 30 min in a 10 mℓ graduate cylinder.

c. Measure settled sludge volume after 30 min.

d. Determine SS (use Step 1).

e. Compute SVI (Reference 3).

5. Ammonia Nitrogen, Distillation and Titration Method, Reference 3, p. 417.

6. Organic Nitrogen, Distillation and Titration Method, Reference 3, p. 437.

7. Total Oxidized Nitrogen, Devawda's Alloy Reduction Method, Reference 3, p. 431.

EXPERIMENT III-B-2

BATCH DETERMINATION OF CELL YIELD IN AEROBIC SYSTEMS

Purpose

The purpose of this experiment is to measure the growth yield for a number of pure substrates and to determine the relationship of growth yield to the theoretical oxygen demand (TOD) of the substrate.

Introduction

When an organic compound (or substrate) can serve as both a carbon source and an energy source for the growth of a population of microorganisms, a portion of the substrate is catabolized to provide energy and the remainder is assimilated to form new protoplasm. The materials balance for the growth of a microbial culture on a single substrate may be expressed as:

Substrate Consumed	=	Substrate Oxidized	+	Substrate Assimilated

The growth yield of a substrate is defined as the amount of new protoplasm (cells) produced per unit of substrate consumed.

For a given substrate, the growth yield should be independent of the substrate concentration as long as the other nutrients are supplied in adequate amounts and the physical environment is not limiting.

Since the amount of "useful" energy required to synthesize a given amount of protoplasm is constant, the growth yield for a particular substrate should be proportional to the free energy of oxidation of the substrate.

The free energy of oxidation varies considerably from one substrate to another but for carbonaceous substrates being utilized by aerobic heterotrophs it is approximately proportional to the amount of oxygen required to oxidize a unit amount of substrate to CO_2 and H_2O. Thus, as a first approximation, the growth yield for a given substrate should be proportional to the total oxygen demand (TOD) of the substrate. The TOD of a substrate is computed from the oxidation reduction equation in which the substrate is oxidized to CO_2 and H_2O by O_2. Thus for glucose, $C_6H_{12}O_6$, the TOD can be computed from:

Molecular weight $C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$

$$\text{TOD glucose} = \frac{192}{180} = 1.067 \frac{\text{mg } O_2}{\text{mg substrate}}$$

Procedure

1. The substrates that will be studied are glucose, sucrose, glutamic acid, sodium tartrate and sodium acetate each at concentrations of 10, 30, 100 and 300 mg/ℓ in a basal salts medium. The source of microorganisms will be settled domestic sewage.
2. Determine the bacterial population in a decanted supernatant of a sample of fresh sewage that has been allowed to settle for 2 hr using the following technique:
   a. Mix culture well, remove 4.5 mℓ, and place in sterile test tube.
   b. Add 0.5 mℓ of the filtered staining solution and mix vigorously.

c. Heat the tube over a flame until it bumps slightly.

d. Remove from heat and allow to cool for 3 min.

e. Place two drops of the stained bacteria suspension in the Petroff-Hauser counting chamber and place the cover slip into position so that no air bubbles are trapped.

f. Place the counting chamber on the stage of a microscope and allow to stand for 5 min.

g. Place a drop of oil on the cover slip and focus on the ruled field with the oil immersion lens.

h. Count the bacteria in each square. Continue counting until at least 100 bacteria have been counted.

j. Calculate the number of bacteria/mℓ from the relationship:

$$\frac{\text{No. bacteria counted}}{\text{No. squares}} \times 2.22 \times 10^7 = \frac{\text{No. bacteria}}{\text{mℓ}}$$

3. Based on the initial bacteria count of the settled sewage make a serial dilution of the sewage in Basal Medium so that the final dilution contains between 10^3 and 10^4 bacteria/mℓ. Use 50 mℓ Basal Medium for serial dilutions.

4. Prepare the following media by adding the following volume of substrate stock solution to 100 mℓ Basal Medium contained in 250 mℓ erlenmeyer flasks:

Stock Substrate Solution mℓ	Desired Substrate Concentration In Basal Medium, mg/ℓ
0	0
10	0.1
30	0.3
100	1
300	3

5. Stopper each flask with cotton and place the cultures on shaker. Incubate at 35 C. Aseptically sample each culture at 12 hr intervals and determine the bacteria count microscopically. After the bacteria count has remained constant for three successive samples, it may be assumed that all of the substrate has been consumed and that growth has ceased and the experiment may be terminated.

Data Analysis

1. Report the bacteria counts for each culture in tabular form.
2. Plot a curve of bacteria counts vs time (growth curve) for each culture and from these curves estimate the maximum bacterial count.
3. Plot the maximum cell count for each substrate vs substrate concentration. Fit a straight line to the points and compute its slope. This value is the growth yield.
4. Calculate the TOD of each substrate and plot growth yield vs substrate TOD. Does the plot appear to be linear?

Apparatus

Pipettes.

Flasks, 50 mℓ.

Shaker Table: To hold 25, 250 mℓ erlenmeyer flasks.

Incubator: 35 C to hold shaker table.

Microscope: Oil immersion objective.

Counting Chamber: Petroff-Hauser.

Erlenmeyer Flasks, 250 mℓ: 25 per student group.

Glass-wool filtered compressed air supply with air stone.

Millipore filter apparatus.

Membrane filters: 0.45 μm47 mm diameter.

Reagents

Basal Medium: Prepare 100 mℓ of each of the following Stock Solutions:

$MgSO_4 \cdot 7H_2O$	20 mg/mℓ
$FeSO_4 \cdot 7H_2O$	20 mg/mℓ
KH_2PO_4	200 mg/mℓ
K_2HPO_4	160 mg/mℓ
NH_4Cl	200 mg/mℓ

Add 1 mℓ of each stock solution to about 250 mℓ distilled water and dilute to 1ℓ. Prepare one day before the experiment and aerate overnight, (3ℓ per student group).

Fresh settled domestic sewage: (50 mℓ per student group).

Saturated alcoholic Methylene Blue: Dissolve 5 g Methylene Blue in 100 mℓ 95% ethyl alcohol, allow to stand overnight, and decant.

Aqueous phenol solution: 5%, dissolve 50 g phenol in distilled water and make up to 1 ℓ.

Staining solution: Mix 1 mℓ of alcoholic Methylene Blue solution with 50 mℓ aqueous phenol solution and filter through an 0.45 µm membrane filter.

EXPERIMENT III-C-1

BIODEGRADABILITY INTRODUCTION

Purpose

The purpose of these experiments is to demonstrate several factors related to the ability of a population of microorganisms to use various substrates in their metabolic processes.

Introduction

The term biodegradability is a relative one since both the rate and extent of breakdown of a particular compound is highly dependent on the system in which its degradation is studied. Thus, the type, number and degree of acclimation of microorganisms and the nature and concentration of nutrients and other substrates besides the material whose biodegradability is being assessed are important factors. The presence of oxygen, the temperature and the method by which degradation is assessed are critical. To be useful in practice a biodegradability test must parallel the practical situation in which the material will be degraded. Thus, if the fate of a material in a stream is of interest a useful method might be the so-called "river die away test" in which the compound is incubated in river water (in a BOD bottle) at the temperature of the stream. Conversely, if the fate of a substance in the activated sludge process is to be determined, it would be pertinent to examine its biodegradability in a laboratory activated sludge unit.

The biodegradability of a substance, by definition, depends on the ability of the biological agencies present to degrade it. Very often it

is observed that the process of "acclimation", in which microorganisms are grown in the presence of the substance under study prior to being used in a biodegradability assessment, will cause a more rapid and/or complete degradation of the substance. This period of "acclimation" may serve to increase the extent or rate of biodegradation by selectively increasing the number of microorganisms capable of utilizing the substrate and allowing time for the development of inducible enzymes needed for substrate degradation.

The assessment of biodegradability depends strongly on the analytical measurement made to determine the residual level of compound or to otherwise measure the course of biodegradation. Thus, in the determination of biodegradation of anionic synthetic detergents, an analytical procedure using Methylene Blue is commonly used to determine residual detergent concentration. This procedure will measure synthetic anionic detergents of the alkyl aryl sulfonate type down to alkyl chain lengths of about C_6. Thus, complete degradation of a C_{14} alkyl aryl sulfonate would be registered by this technique when the alkyl chain had been reduced to a length of less than 6 carbon units.

In aerobic systems one commonly utilized index of the biodegradation of a substrate is the stimulation of oxygen consumption in the presence of the material. This is an indirect measurement because it records the additional consumption of an electron acceptor in the presence of the substrate under study. It is not possible to exactly determine the extent of degradation from oxygen uptake measurements because one is never certain what fraction of the substance is being catabolized and what amount is being synthesized into cell material.

However, such experiments yield useful data on relative rates of biodegradation and, if coupled with measurements of substrate concentration, can yield exact information on the extent of degradation.

The experiments in this section are designed to illustrate the effect of chemical structure on biodegradation and the effect of acclimation of microorganisms on biodegradation.

The experiment on primary, secondary and tertiary alcohols (Experiment III-C-2) illustrates the effect of chemical structure on biodegradation of a series of isomers. Oxygen uptake is used as an index of biodegradation. The experiment on hard and soft detergents (Experiment III-C-3) serves both to illustrate the effect of chemical structure on biodegradation as well as to demonstrate the phenomenon of acclimation. Both the disappearance of detergent and the uptake of dissolved oxygen are used as indices of degradation. The experiment on oxygen uptake in the presence of glucose, galactose and lactose (Experiment III-C-4) also demonstrates the effect of acclimation of mixed microbial cultures on substrate biodegradation.

Experiment III-C-2

BIODEGRADATION OF BUTYL ALCOHOLS

Purpose

To demonstrate the effect of branching of an aliphatic alkyl chain on rate and extent of aerobic biodegradation of alcohols.

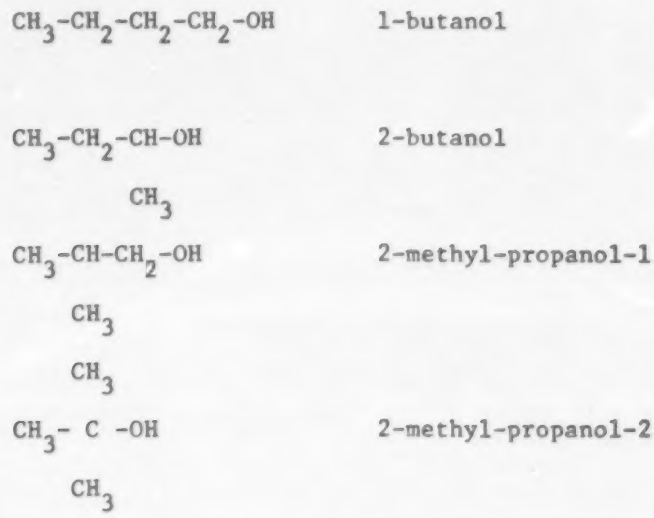
References

1. Hammerton, C. "Observations on the Decay of Synthetic Anionic Detergents in Natural Waters." J. Appl. Chem., 5:517, 1955.
2. Standard Methods for the Examination of Water and Wastewater, 14th ed. American Public Health Association, 440, 1976.

Introduction

Normal straight chain aliphatic hydrocarbons are commonly oxidized by a stepwise attack from the chain terminus that involves first the terminal (or ω) oxidation of the chain to a carboxyl group then usually the stepwise removal of C_2 units by so-called β-oxidation. If the alkyl chain contains branches (e.g. one or more methyl groups on a side chain) then β-oxidation may be hindered. If a quaternary carbon (a carbon with no hydrogens attached to it) is present at the site of β-oxidation this mechanism will not proceed because one of the intermediate steps in the cleavage of C_2 units by β-oxidation is the formation of a C=C bond, which cannot take place at a carbon bonded to 4 other carbons. In this experiment four C_4 alcohols which have varying types and degrees of chain branching will be incubated with BOD dilution water and the oxygen

uptake due to the presence of the alcohol will be followed as an index of biodegradation. The compounds are:



Procedure

1. Prepare fresh 15ℓ BOD dilution water (Reference 2, p. 546).
   Aerate for 1 hr to saturate with dissolved oxygen.
   Add 30 mℓ settled sewage seed. Mix thoroughly by aeration.
2. Proportion seeded BOD dilution water into four 2ℓ aliquots labelled A,B,C and D.
   To aliquot A add 20 mℓ, 300 mg/ℓ 1-butanol solution.
   To aliquot B add 20 mℓ, 300 mg/ℓ 2-butanol solution.
   To aliquot C add 20 mℓ, 300 mg/ℓ 2-methyl-propanol-1 solution.
   To aliquot D add 20 mℓ, 300 mg/ℓ, 2-methyl-propanol-2 solution.
   Mix in the alcohol solution carefully by gentle stirring to avoid evaporation.
3. From each of the aliquots A,B,C, and D fill five, 300 mℓ BOD bottles, stopper and incubate at 20 C immediately.

4. Proportion the remaining seeded aerated dilution water into 12, 300 mℓ BOD bottles and incubate 10 of them immediately. These bottles will serve as "blanks" to determine the initial DO and the oxygen uptake of the seeded dilution water.

5. Determine at once the initial DO on the two unincubated bottles.

6. Remove BOD bottles from the 20 C incubator on the following schedule and determine DO concentration using the azide modification of the Winkler Method as in Reference 2, p. 443.

Time of Incubation, Days	Blank	Aliquot A	Aliquot B	Aliquot C	Aliquot D
0	X				
1	X	X	X		
1.5	X	X	X		
2	X	X	X	X	
3	X	X	X	X	X
4	X	X	X	X	X
5	X			X	
7	X			X	X
9	X				X
11	X				X
13	X				X

X Remove from incubator

Data Analysis

1. Compute the DO uptake due to the presence of the four C_4 alcohols at each sampling time by subtracting the DO uptake of the seeded dilution water from the total DO uptake.
2. Present the DO uptake due to each of the C_4 alcohols vs time in graphical form.
3. What is the effect of various types of alkyl chain branching on the extent and rate of biodegradation of C_4 alcohols?
4. Using the maximum DO uptake due to the presence of each alcohol, what is the greatest percent of theoretical oxidation of each alcohol obtained during this experiment?
5. Present a detailed mechanistic pathway for β-oxidation of aliphatic hydrocarbon chains. What other oxidation mechanisms are common in microorganisms for degrading aliphatic hydrocarbon chains?

Apparatus

A darkened air incubator or water bath: 20 C ± 1 C.

BOD bottles, 300 mℓ: 32 per student group.

Glass bottle capable of holding 15ℓ.

Compressed air supply fitted with glass wool filter and air stone for aerating BOD dilution water.

Reagents

Dissolved Oxygen Determination:

Manganous sulfate solution: Dissolve 480 g $MnSO_4 \cdot 4H_2O$ or 400 g $MnSO_4 \cdot 2H_2O$ or 364 g $MnSO_4 \cdot H_2O$ in distilled water, filter, and dilute to 1ℓ; approximately 70 mℓ/student group.

Alkali-iodide-azide reagent: Dissolve 500 g sodium hydroxide, NaOH, and 135 g sodium iodide, NaI, in distilled water and dilute to 1ℓ. To this solution add 10 g sodium azide, NaN_3, dissolved in 40 mℓ distilled water; approximately 70 mℓ/ student group.

Sulfuric acid, conc.: approximately 70 mℓ/student group.

Sulfuric acid, approximately 3.6 N. Dilute 1 volume conc. H_2SO_4 with 9 volumes of distilled water; approximately 70 mℓ/student group.

Starch solution: Prepare an emulsion of 5-6 g potato, arrowroot, or soluble starch in a mortar or beaker with a small quantity of distilled water. Pour this emulsion into 1ℓ of boiling water, allow to boil a few minutes, and let settle overnight. Use the clear supernatant; approximately 70 mℓ/student group.

Sodium thiosulfate stock solution, 0.1C N: Dissolve 24.82 g $Na_2S_2O_3 \cdot 5H_2O$ in distilled water and dilute to 1ℓ. Preserve by adding 5 mℓ chloroform.

Standard sodium thiosulfate titrant, 0.025 N: Dilute 250 mℓ sodium thiosulfate stock solution to 1ℓ. Standardize with potassium biniodate; approximately 200 mℓ/student group.

Standard potassium biniodate solution, 0.025 N: Dissolve exactly 0.8124 g $KH(IO_3)_2$ in distilled water and dilute to 1ℓ. approximately 100 mℓ/student group.

Standardization of sodium thiosulfate titrant: Dissolve approximately 2 g KI in 100 to 150 mℓ distilled water; add 10 mℓ approximately 3.6 N H_2SO_4 followed by exactly 20 mℓ standard biniodate

solution. Dilute to 200 mℓ and titrate with thiosulfate, adding starch toward the end of the titration when a pale straw color is reached.

BOD dilution water:

Distilled water.

Phosphate buffer solution: Dissolve 8.5 g KH_2PO_4, 21.75 g K_2HPO_4, 33.4 g $Na_2HPO_4 \cdot 7H_2O$, and 1.7 g NH_4Cl in approximately 500 mℓ distilled water and dilute to 1ℓ. The pH of this buffer should be 7.2.

Magnesium sulfate solution: Dissolve 22.5 g $MgSO_4 \cdot 7H_2O$ in distilled water and dilute to 1ℓ.

Calcium chloride solution: Dissolve 27.5 g $CaCl_2$ in distilled water and dilute to 1ℓ.

Ferric chloride solution: Dissolve 0.25 g $FeCl_3 \cdot 6H_2O$ in distilled water and dilute to 1ℓ.

Settled sewage seed: Store settled domestic sewage for 48 hr in a 20 C incubator.

Dilution water: Place the desired volume of 20 C distilled water in a suitable bottle and add 1 mℓ each of phosphate buffer, magnesium sulfate, calcium chloride, and ferric chloride solution for each liter of water. Aerate or shake to saturate with DO. Just prior to use, add required volume of seed; approximately 15ℓ dilution water per student group.

C_4 alcohol solutions, 300 mg/ℓ each: Prepare immediately before use in distilled water. Using a micro-pipette add the following volumes of C_4 alcohols to separate 1ℓ batches of distilled water.

1-butanol	0.37 mℓ
2-butanol	0.37 mℓ
2-methyl-propanol-1	0.38 mℓ
2-methyl-propanol-2	0.38 mℓ

Mix carefully by swirling in a closed bottle. Use at once and discard remaining solution.

EXPERIMENT III-C-3

BIODEGRADATION OF ALKYL ARYL SULFONATES

Purpose

This experiment on biodegradation under "river die away" conditions is divided into two parts. In the first section the biodegradability of alkyl aryl sulfonates with a high and low degree of alkyl chain branching is compared. In the second section the effect of acclimation on the rate and extent of biodegradation of a sparingly-branched alkyl aryl sulfonate is studied.

References

1. Swisher, R.D. Surfactant Biodegradation, Marcel Dekker, New York, 1970.
2. Standard Methods for the Examination of Water and Wastewater, 14th ed. American Public Health Association, 1976.
3. Hammerton, C. "Observations on the Decay of Synthetic Anionic Detergents in Natural Waters", J. Appl. Chem., 5:517, 1955.

Introduction

One of the factors that has been found to strongly influence the rate and extent of degradation of alkyl aryl sulfonates is the frequency and type of branching in the alkyl chain. Evidence exists to support the contention that these anionic detergents are degraded by an attack on the alkyl chain starting with ω-oxidation of the terminal carbon to a carboxyl group followed by β-oxidation of the chain down to the vicinity

of the benzene ring. Both of the anionic detergents used in this experiment are mixtures of many isomers which the Methylene Blue test does not differentiate. The results must therefore be interpreted with caution if statements are to be made on the relative degradability of various chemical structures. It should be possible however to generally conclude which mixture of isomers - the sparingly-branched LAS or "linear alkyl sulfonate" or the more highly-branched TPBS or tetra-propylene-benzene-sulfonate is more degradable. Destruction of the benzene ring with release of inorganic sulfate has been shown to occur as a futher step in the microbial degradation of these anionic detergents.

The commonly-used Methylene Blue analysis for anionic detergents is capable of detecting alkyl aryl sulfonates that have chain lengths of greater than about six carbons, and not capable of detecting residues of these compounds of any chain length once the sulfonate group has been removed. Thus, if this analytical test is used to assess alkyl aryl sulfonate biodegradation then the results will not necessarily compare exactly with those derived from measurements of DO uptake.

A. Comparison of LAS and TPBS Biodegradability

Procedure

1. Prepare 10ℓ aerated 20 C BOD dilution water as in Experiment III-C-2. Immediately before the experiment add 20 mℓ settled sewage seed and mix by aeration. Split the 10ℓ of seeded dilution water into the three aliquots of 3ℓ, 3.5ℓ and 3.5ℓ respectively.
2. Using the 3ℓ aliquot of seeded BOD dilution water fill 7, 300 mℓ BOD bottles. These are the blanks.

3. To one of the 3.5ℓ aliquots add 35 mℓ stock LAS solution (1g LAS/ℓ) and mix well to give an LAS concentration of 10 mg/ℓ in the diluted mixture. Using the LAS-containing dilution water fill 10, 300 mℓ BOD bottles. These are the LAS Samples.

4. To the second 3.5ℓ aliquot of BOD dilution water add 35 mℓ stock TPBS solution (1g TPBS/ℓ) and mix well to give a TPBS concentration of 10 mg/ℓ in the diluted mixture. Using this mixture fill 10, 300 mℓ BOD bottles. These are the TPBS Samples.

5. Using the Winkler method (Experiment III-C-2) determine immediately the DO on a blank, an LAS sample and a TPBS sample.

6. Using the Methylene Blue method (Reference 2, p. 600) determine the Methylene Blue Active Substance (MBAS) concentration in one BOD bottle from each of the LAS and TPBS samples.

7. Incubate the remaining BOD bottles in the dark at 20 C.

8. Withdraw samples from the incubator for DO and/or MBAS analysis according to the following schedule.

Time of Incubation, hr	Blank DO	LAS Sample		TPBS Sample	
		DO	MBAS	DO	MBAS
0*	X	X	X	X	X
50	X	X		X	
100	X	X	X	X	X
150	X	X		X	
200	X	X		X	
250	X	X		X	
300	X	X	X	X	X

* Initial sample described above prior to incubation.

Data Analysis

1. Present DO uptake and MBAS data in a tabular form.
2. Plot the course of DO uptake of blank, LAS samples and TPBS samples with time. From these plots derive curves that show the course of DO uptake due to LAS and TPBS with time.
3. Plot the course of LAS and TPBS biodegradation as assessed by MBAS concentration with time.
4. Using your knowledge of microbial oxidation and synthesis reactions and the limitations of the Methylene Blue method for anionic detergents, what conclusions can you make on the extent and rate of biodegradation of LAS and TPBS? What further information would be useful in refining this assessment?

Apparatus

Incubator or water bath: 20 C dark.

Compressed air supply with glass wool filter and aeration stone.

Glass carboy: 10ℓ, 1 per student group.

Glass carboy: 4ℓ, 2 per student group.

BOD bottles: 300 mℓ capacity, approximately 30 per student group.

Separatory funnels with teflon stopcocks 500 mℓ approximately 6 per student group.

Spectrophotometer to read at 652 nm with a 1 cm cell.

Reagents

BOD dilution water: See Experiment III-C-2.

Settled Sewage

DO reagents: See Experiment III-C-2.

LAS* stock solution 1.0 g/ℓ. Determine the active material content of the LAS sample and dissolve an amount sufficient to produce a concentration of 1.0 g/ℓ in 50 mℓ distilled water and dilute to 1ℓ.

TPBS* stock solution 1.0 g/ℓ. Determine the active material content of the TPBS sample and dissolve an amount sufficient to produce a concentration of 1.0 g/ℓ in 50 mℓ distilled water and dilute to 1ℓ.

B. Effect of Acclimation on Extent and Rate of LAS Biodegradation

Procedure

1. Prepare 10ℓ aerated 20 C BOD dilution water as in Experiment III-C-2. Immediately before the experiment add 20 mℓ settled sewage seed and mix by aeration. Split the 10ℓ of seeded dilution water into aliquots of 2ℓ and 8ℓ.
2. Using the 2ℓ aliquot of seeded BOD dilution water fill 6, 300 mℓ BOD bottles. These are the blanks.
3. To the 8ℓ aliquot add 40 mℓ stock LAS solution and mix well to give an LAS concentration of 5 mg/ℓ in the diluted mixture. Using the LAS-containing dilution water fill 8, 300 mℓ BOD bottles and 4, 1ℓ bottles.
4. Using the Winkler Method (Experiment III-C-2) determine immediately the DO on two of the 300 mℓ bottles containing LAS. LAS interferes with the Winkler DO titration, causing a sluggish change of the starch iodine end point from blue through purple to pink. To

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* LAS and TPBS can be obtained from the Soap and Detergent Association, 485 Madison Ave., New York, N.Y. 10016

minimize this interference the starch solution should be added at the very last possible point before the end point. This procedure reduces the interference but the end point is still not as sharp as normal. The procedure of performing initial DO determinations on LAS-containing samples is followed in an attempt to cancel out this interference (and any error it produces in DO values by "difference" between an LAS-containing sample and an LAS-containing blank).

5. Incubate the remainder of the samples at 20 C and monitor the progress of DO uptake and LAS disappearance at 2-day intervals using the following procedure:

   a. Saturate about 1ℓ distilled water with dissolved oxygen by aerating for at least 15 min. Determine the DO content of this water using the Winkler Method.

   b. Remove one blank bottle and one 300 mℓ LAS containing bottle from the incubator and carefully withdraw 100 mℓ from each. Set this aliquot aside for determination of surfactant concentration using the Methylene Blue Method.

   c. Carefully replace the 100 mℓ withdrawn for surfactant analysis with the oxygen saturated distilled water prepared in Step 5a. Stopper the bottles and mix several times by inversion.

   d. Determine the DO concentration of the two bottles containing the mixture of incubated sample (and blank) and make-up water. Calculate the DO concentration of the original incubated samples based on the proportion and DO of the make-up water and the determined DO of the mixture.

6. Using the Methylene Blue method determine the LAS concentration

using a suitable aliquot of the 100 mℓ sample withdrawn in Step 5b. The sample for MBAS analysis can be preserved by addition of 50 mg/ℓ mercuric chloride or by freezing.

7. After 12 days (or before, if the DO in the incubated samples becomes exhausted) combine the contents of 4, 1ℓ bottles and aerate at 20 C for at least 30 min to saturate with DO.

8. Split the aerated solution into 2, 2ℓ aliquots. To the first 2ℓ aliquot add 10 mℓ stock LAS solution. Mix by aeration and distribute into 6, 300 mℓ BOD bottles. These are the acclimated seed LAS samples. The second 2ℓ aliquot is introduced into 6, 300 mℓ BOD bottles without further treatment (these are the blanks).

   Immediately determine LAS and DO on one bottle from the acclimated seed, one bottle from the LAS samples and one bottle from the blank series using the procedures outlined in Step 5.

9. Incubate the remaining bottles at 20 C and monitor the DO and LAS concentrations at 2-day intervals for 10 days or more frequently for a shorter period if it appears that the samples are biodegrading more rapidly.

Data Analysis

1. Present data in a concise tabular and graphical form.
2. What effect does acclimation have on the rate and extent of LAS degradation?
3. Is there any evidence that LAS is being oxidized more completely to CO_2 and water by the acclimated rather than the unacclimated seed?
4. Describe a more rapid technique that may be used for acclimating wastewater microorganisms to LAS.

Apparatus

Incubator, 20 C, dark.

BOD bottles: 300 mℓ capacity, approximately 30 per student group.

Glass carboy: 10ℓ, 1 per student group.

Glass carboy: 5ℓ, 2 per student group.

Glass bottles with ground glass stoppers: 1ℓ, 4 per student group.

Compressed air with glass wool filter and air stone.

Reagents

Winkler DO test (See Experiment III-C-2).

Methylene Blue test (Reference 2, p. 600).

BOD dilution water (See Experiment III-C-2)

Settled domestic sewage.

EXPERIMENT III-C-4

ADAPTATION OF MIXED CULTURES - THE BIO-OXIDATION OF MONO- AND DI-SACCHARIDES

Purpose

The objective of this experiment is to demonstrate the ability of a mixed culture of microorganisms to adapt to the utilization of a carbon source. Specifically the ability as measured by dissolved oxygen uptake of acclimated and unacclimated cultures to degrade lactose will be studied.

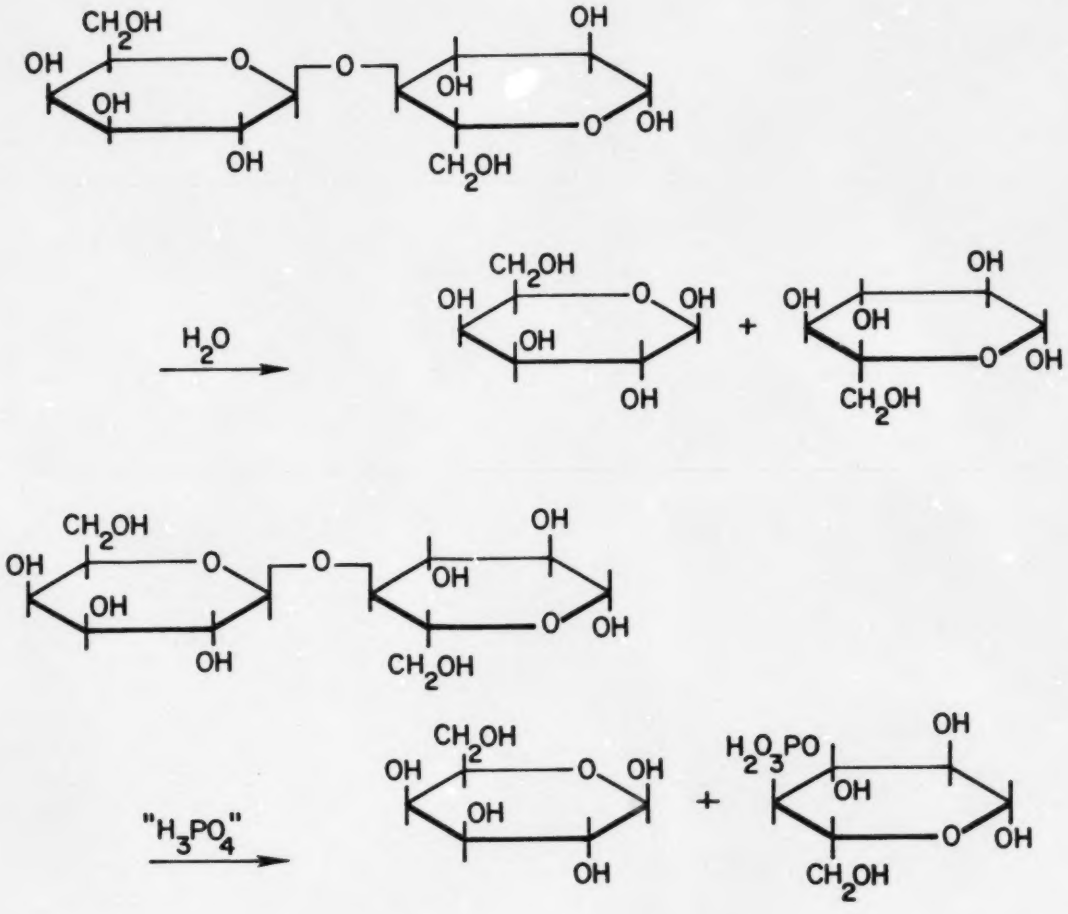
References

1. Mahler, H.R. and Cordes, E.H. Biological Chemistry, Harper and Row, 1966.
2. Standard Methods for the Examination of Water and Wastewater, 14th ed. American Public Health Association, 1976.

Introduction

Metabolic pathways are largely determined by both the requirements of a microorganism and its surrounding environment. Since metabolic pathways are dictated by the enzymic makeup of the microorganisms, regulation of the nature and of the rate of enzymes is an important factor in determining the metabolic capability of microorganisms. There are two general ways in which metabolism can be regulated - these are by regulating rates using feedback control or by allowing the medium to exert an influence on the rate and/or extent of enzyme formation. The

latter mechanism is referred to as induction when the effect is positive and repression when the effect is negative. In this experiment the reaction of interest is the hydrolysis of the disaccharide, lactose, into its component monosaccharides, D-galactose and D-glucose.



This experiment will not differentiate between these reactions since it measures only the end result of the relative availability of a substrate for energy metabolism in an aerobic environment i.e., oxygen uptake rate. Because of the use of a mixed microbial culture in this experiment, it will not be possible to conclude whether lactose hydrolysis is a result of enzyme induction in a single organism or selection of a lactose hydrolysing microorganism from the mixed population of microorganisms.

The experiment consists of following the dissolved oxygen uptake of BOD dilution water that has been seeded with sewage unacclimated to lactose and with supernatant from a fill-and-draw activated sludge unit that has been operated in the presence of lactose. Differences in the dissolved oxygen uptake curves are interpreted in terms of the ability of these two mixed cultures to metabolize glucose, galactose and lactose solutions.

Procedure

1. Prepare 20 ℓ of BOD dilution water as described in Experiment III-C-2 and aerate continuously for at least 1 hr to saturate with dissolved oxygen. Immediately prior to the sample preparation withdraw a 3ℓ aliquot of this dilution water and add to it 6 mℓ lactose acclimated seed. To the remaining 17ℓ of dilution water add 34 mℓ settled domestic sewage seed. Mix both of the seeded aliquots completely by aerating.
2. Prepare 10 mg/ℓ solutions of glucose, galactose and lactose in sewage-seeded dilution water as follows:
   a. Add 30 mℓ of 1 mg/mℓ freshly-prepared glucose stock solution

to a 4ℓ container and dilute to 3ℓ with sewage-seeded dilution water.

b. Add 30 mℓ of 1 mg/ℓ freshly-prepared galactose stock solution to a 4ℓ container and dilute to 3ℓ with sewage-seeded solution water.

c. Add 30 mℓ of 1 mg/mℓ freshly-prepared lactose stock solution to a 4ℓ container and dilute to 3ℓ with sewage-seeded dilution water.

3. Add 30 mℓ of a 1 mg/mℓ freshly-prepared lactose stock solution to a 4ℓ container and dilute to 3ℓ with dilution water seeded with lactose-acclimated microorganisms.

4. Proportion each of the four 3ℓ aliquots into a series of 8, 300 mℓ BOD bottles. Stopper securely and incubate in the dark at 20 $\pm$ 1 C.

5. Use the remaining sewage-seeded dilution water to fill 10, 300 mℓ BOD bottles. Stopper these securely. These are the blanks. Incubate 8 blank bottles at 20 C. On the two remaining blank bottles determine the initial dissolved oxygen concentration using the azide modification of the Winkler Method (Reference 2, P. 443).

6. At daily intervals for one week withdraw one blank and one of each of the samples from the incubator and determine the dissolved oxygen concentration in each. If it appears from the data that important differences in dissolved oxygen uptake are taking place that would possibly be missed by waiting for the next day's sample, the sampling can be made more frequent than a 24 hr interval.

Data Analysis

1. Graphically summarize the DO uptake with time for each of the samples. Comment on any differences between the uptake curves and between the different seeds for the lactose solution.
2. Compute the theoretical oxygen demand for the glucose and lactose oxidations. What percentage of theoretical oxidation of each sugar is attained in this experiment? Comment on the magnitude of this value.

Apparatus

BOD bottles: 300 mℓ with tapered glass stoppers, 34 per student group.

Erlenmeyer flasks: 4ℓ capacity, 3 per student group.

Glass carboy: 5 gal.

Incubator, 20 C ± 1 C.

Reagents

Distilled water.

Stock lactose solution, 1 mg/mℓ: dissolve 1.00 g lactose in distilled water and make up to 1000 mℓ; prepare fresh daily.

Stock glucose solution, 1 mg per mℓ: dissolve 1.00 g D-glucose in distilled water and make up to 1000 mℓ; prepare fresh daily.

Stock galactose solution, 1 mg/mℓ: dissolve 1.00 g D-galactose in distilled water and make up to 1000 mℓ; prepare fresh daily.

Settled sewage seed: store settled domestic sewage for 24-48 hr in a 20 C incubator.

Reagents for dilution water: see Experiment III-C-2.

Sewage seeded BOD dilution water: see Experiment III-C-2.

Lactose acclimated seed: develop a lactose-oxidizing seed by aerating a 1ℓ sample of activated sludge (MLSS of approximately 1500 mg/ℓ) with settled sewage to which has been added 100 mg/ℓ lactose. After 23 hr aeration allow the mixed liquor to settle for 1 hr. Decant the supernatant liquor and make up to the sludge portion to 1ℓ with settled domestic sewage to which 100 mg lactose has been added immediately prior to feeding. Continue the aeration and feeding in a 24 hr cycle for at least 2 weeks. Two days prior to the start of the experiment place the supernatant from this unit in the 20 C incubator. Use 2 mℓ/ℓ of this material to seed dilution water.

EXPERIMENT III-D-1

TOXICITY OF HEAVY METALS TO MIXED MICROBIAL CULTURES

Purpose

The objective of this experiment is to investigate the toxicity of two metallic salts to mixed microbial cultures. The toxic effect will be assessed by a suppression of rate and extent of dissolved oxygen uptake over a culture incubated in the absence of the metallic salts.

References

1. Standard Methods for the Examination of Water and Wastewater, 14th ed. American Public Health Association, p. 443, 1976.

Theory

The toxic action of heavy metals varies with both the specific metal, the concentration of metal and the chemical composition of the medium (which may influence the speciation of the metal). Effects for individual metals range from complete loss of viability and inhibition of metabolic function through retardation of metabolism and growth to "no effect" and stimulation of these functions. In this experiment the metals, mercury (as mercuric chloride) and zinc (as zinc sulfate) will be added to a mixed culture of microorganisms in concentrations that are designed to illustrate the entire range of effects enumerated above as depicted by dissolved oxygen uptake rate.

Procedure

A. Mercuric Chloride

1. Prepare 20ℓ aerated BOD dilution water (see Experiemnt III-C-2).

2. To the 20ℓ aerated dilution water add settled sewage at a rate that would produce an oxygen uptake of 7 mg/ℓ over a period of 2 weeks. For a typical domestic settled sewage with an ultimate carbonaceous oxygen demand of 300 mg/ℓ an appropriate dilution would be 700 mℓ settled sewage/20ℓ dilution water.

3. Prepare a series of mercuric chloride solutions in settled sewage seeded dilution water that contain 10, 1, 0.5, 0.1 and 0.01 mg Hg^{2+}/mℓ using the following procedure:

   a. To a 4ℓ container add 40 mℓ stock (1 mg Hg^{2+}/mℓ) mercuric chloride solution and dilute to 4ℓ with sewage seeded dilution water. This is Solution I and contains 10 mg Hg^{2+}/ℓ. Use Solution I to fill 8, 300 mℓ-BOD bottles.

   b. With the remaining volume of Solution I prepare Solutions II, III and IV by adding respectively 300 mℓ, 150 mℓ and 30 mℓ of Solution I to separate 4ℓ containers and diluting to 3ℓ with sewage seeded dilution water. These solutions contain 1.0, 0.5, and 0.1 mg Hg^{2+}/ℓ respectively. Fill 8, 300 mℓ BOD bottles from each of these solution.

   c. With the remaining volume of Solution II prepare Solution V by adding 30 mℓ of Solution II to a 4ℓ container and diluting to 3ℓ with sewage-seeded dilution water. Fill 8, 300 mℓ BOD bottles with Solution V.

4. Prepare a series of 10, 300 mℓ BOD bottles with sewage seeded dilution water alone. These are the blanks.
5. Stopper all filled BOD bottles excluding air and incubate at 20 C ± 1 C in the dark.
6. Immediately determine the dissolved oxygen concentration in two of the blank bottles using the azide modification of the Winkler Method (Reference 1, p. 443).
7. At intervals of 48 hr over a 2 week period remove one blank bottle and one bottle from each of Solutions I-V from the incubator and perform a dissolved oxygen determination on each.

B. Zinc Sulfate

1. Prepare 20ℓ of sewage seeded aerated BOD dilution water as in Steps A1 and A2.
2. Prepare a series of solutions containing a range of zinc concentrations by serial dilution as described in Step A3 above. Concentrations of 1, 10, 50, 100, 500 mg Zn/ℓ are suggested.
3. Prepare blanks as in Step A4 above.
4. Follow the dissolved oxygen uptake in these samples and blanks as in Steps A5, A6 and A7 above.

Data Analyses

1. Present the data for each metallic salt in tabular and graphical format.
2. From these data what would you judge to be the toxic level of each of these metals?

3. From your data what is the "no effect" level for each of these metals?

4. How might a procedure such as the one performed here be used as a routine test for evaluating toxicity of waste streams or sewage treatment plant effluents? Would such a test be practical? How could the results be applied?

Apparatus

BOD bottles; 300 mℓ capacity with tapered glass stoppers, 100 per student group.

Erlenmeyer flasks; 4ℓ capacity, 10 per student group.

Glass Carboy; 5 gal.

Incubator, 20 $\pm$ 1 C.

Reagents

Stock Hg^{2+} solution, 1 mg Hg^{2+}/mℓ: Add 1.360 g, 99.5% purity $HgCl_2$ to a 1ℓ volumetric flask and make up to the mark; approximately 100 mℓ per student group.

Settled sewage seed; store settled domestic sewage for 24-48 hr at 20 C.

Reagents for dilution water: See Experiment III-C-2.

Reagents for dissolved oxygen determination: See Experiment III-C-2.

Sewage seeded BOD dilution water: See Experiment III-C-2, approximately 40ℓ per student group.

Stock Zn^{2+} solution, 1 mg Zn^{2+}/mℓ; add 4.395 g $ZnSO_4 \cdot 7H_2O$ to a 1ℓ volumetric flask and make up to the mark; approximately 100 mℓ per student group.

EXPERIMENT III-E-1

REMOVAL OF BACTERIA AND BACTERIOPHAGE BY RAPID SAND FILTRATION

Purpose

To demonstrate the effectiveness of rapid sand filtration in removing bacteria and viruses from drinking water.

References

1. Rich, L.G. Environmental Systems Engineering, McGraw Hill, Inc., N.Y. p. 371, 1973.
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Introduction

Most modern municipal water treatment plants processing surface waters for municipal supply employ a process train of coagulation, flocculation, sedimentation and rapid sand filtration. While it has been well demonstrated that the rapid sand filtration step is effective in removing suspended matter including bacteria and viruses it is known that the efficacy of removal of microorganisms depends to a large degree upon the chemical coagulation step and the rate at which the resultant water is filtered. Coagulation/flocculation is commonly performed by the addition of aluminum or iron salts to the water resulting in the formation of a hydrous metal oxide floc. It is during the formation of these flocs that bacteria and viruses are enmeshed and sorbed to the floc surfaces thus enabling their removal by subsequent sedimentation and rapid sand filtration.

This experiment is designed to demonstrate the effectiveness of chemical flocculation and sand filtration in removing microorganisms from water.

Procedure

1. Place 15 or 20 glass beads in the bottom of each of two 2 ℓ percolator funnels (Corning No. 740 or equivalent). Cover the glass beads with a small amount of glass wool. Fill each funnel 2/3 full with coarse sand. To the bottom of the funnel attach a short section of rubber hose and close with a pinch cock.
2. Cover each funnel unit with aluminum foil and autoclave at 121 C for 20-25 min.
3. Set the two sterile filtration units into ring stands and pour 500 mℓ sterile distilled water into each. Drain off enough water so that the sand layer is saturated with water. (Leave a depth of about 1 mm of water on top of the sand column). Label the filter units A and B.
4. The water to be tested consists of distilled or tap water adjusted to pH 6.0 (1 mℓ/ℓ standard buffer) and seed at the rate of 1 mℓ/ℓ with an 18 hr culture of a T 4 phage resistant E. coli. This should give a test water containing approximately 3×10^9 cells/mℓ. The test water is also seeded with 1 mℓ of a high titer culture (approximately 1×10^9 plaque forming units, pfu/mℓ) of bactriophage T4 (ATCC11303-B4).
5. Take two 500 mℓ portions of seeded water and label them A and B. From sample A aseptically remove a 1.0 mℓ portion and prepare

serial 10-fold dilutions in sterile dilution buffer using 9 mℓ dilution blanks. The dilutions should cover the range from 10^{-2} to 10^{-4}.

6. Plate 0.1 mℓ of each dilution prepared in Step 5 above using the spread plate technique as follows: place 0.1 mℓ sample or dilution on to the surface of a nutrient agar culture plate (use standard petri dish) and spread the liquid over the agar surface using a bent glass rod. Sterilize the glass rod by immersing in alcohol and igniting in a burner. Cool before use. Invert the plates and incubate for 24 hr at 35 C.

7. Precoat an 0.45 μm Swinnex Millipore or equivalent filter by filtering 3 mℓ of a sterile peptone solution. Withdraw 10 mℓ from water sample A with a sterile 10-15 mℓ syringe and filter through the precoated 0.45 μm filter. Collect the filtrate in a sterile screw-capped test tube. This step separates the bacteriophage from the bacteria. Make 10-fold dilutions of this filtrate in the range from 10^{-2} to 10^{-4} using sterile dilution buffer.

8. Perform a bacteriophage assay from each of the dilutions in Step 7 as follows: To a tube of 3 mℓ soft LM agar held at 44-45 C, add 1-2 drops of 18 hr culture of the host bacterial strain (the E. coli ATCC 1103) and subsequently add 0.1 mℓ phage filtrate or dilution. Prepare one tube for each dilution. Immediately pour the contents of the tube onto the surface of a previously prepared culture plate of LCM agar and gently rock the plate to spread the soft agar evenly. When the agar layer has solidified (allow about 20 min), incubate at 30 C for 18 hr. Refrigerate the plates at the end of

the incubation period. The number of phage present are equivalent to the number of clear plaques formed on the agar surface.

9. Pour the remainder of sample A through the sand filter labeled A and collect the last 25 to 50 mℓ filtrate in a sterile 100 mℓ erlenmeyer flask. Repeat the assay procedures described in Step 6, 7 and 8 on this filtrate.

10. To the second 500 mℓ sample of seeded test water (sample B), add 15 mℓ of a 5% w/v solution of $Al_2(SO_4)_3 \cdot 18H_2O$ and 2.5 mℓ of 2M Na_2CO_3. These two solutions should be added slowly and at the same time so that a good floc will form but the pH of the solution will not vary too greatly. Stir the mixture slowly with a magnetic stirrer for about 5 min to form a heavy floc. Allow to settle for 30 min. Assay the supernatant for E. coli and bacteriophage using the methods described above.

11. Pour the clear supernatant through the second sand filter and collect the last 25-50 mℓ in a sterile 100 mℓ erlenmeyer flask. Assay this filtrate for E. coli and bacteriophage as above.

Results

1. Record the data in tabular form indicating the number of bacteria or bacteriophage present in the unfiltered water, the numbers remaining after filtration without prior coagulation, the number remaining after filtration with prior coagulaiton and the numbers removed by the coagulation step alone.

2. Report the removals in terms of percent of original numbers.

3. Record the filtration flow rates and comment upon the efficiency of

microorganism removal by filtration relative to the removal efficiency by flocculation alone.

4. Compare the results obtained by other student groups in the class.

Apparatus

Rapid sand filter assemblies consisting of a 2 ℓ percolator funnel (Corning No. 740), clean coarse sand, (effective size approximately 0.05 cm, uniformity coefficient of approximately 1.5), rubber tubing, glass wool, ring stand and ring and glass beads, 2 per student group.

Sterile swinnex membrane filter, 0.45 μm: Millipore filter corporation or equivalent, 4 per student group.

Lurelock syringes: sterile, 10-15 mℓ; 4 per student group.

Hypodermic needles: sterile, 1 in., 20 guage; 4 per student group.

Screw cap culture tubes: 13 mm x 100 mm, sterile; 3 per student group.

Dilution blanks: 9 mℓ; 30 per student group.

Pipettes: 1 mℓ, 10 mℓ, sterile.

Reagents

1% w/v Bacto-peptone: Difco.

5% w/v solution of $Al_2(SO_4)_3 \cdot 18H_2O$; 20 mℓ per student group.

2M Na_2CO_3, dissolve 21.2 g Na_2CO_3 in distilled water and dilute to 100 mℓ; 5 mℓ per student group.

Escherichia coli B: American Type Culture Collection, ATCC 11303.

Bacteriophage T4 ATCC 11303-B4.

Bacteriophage seed: Inoculate 3, 50 mℓ erlenmeyer flasks containing 10 mℓ LCM broth with 2 mℓ of an exponentially growing culture of E. coli B. (about 10^9 cells/mℓ). Subsequently, add one drop of bacteriophage suspension (suspended in LCM Broth) to each of two flasks. The third flask should be maintained as a control. Incubate at 35 C and examine periodically for about a 3-hour period. Actively growing bacteriophage will lyse the bacterial culture. A lysed culture will be clear when held against the light. If both cultures lyse the one which takes the longest for lysis will have the highest phage titer. Centrifuge 10 mℓ of lysed culture at approximately 7000 XG for 20 min to remove cell debris. Decant the supernatant liquid into a sterile, screw-capped tube; add 1 mℓ chloroform, shake well and allow to stand at room temperature for 20 min and then store in the refrigerator until used. Assay using the phage soft agar technique discribed in Step 8 of procedure. Phage titer should be in the range of 10^9 to 10^{11} pfu/mℓ.

Bacteriophage resistant E. coli B: To a tube of 3 mℓ soft LM agar held at 44-45 C, add 1-2 drops of an 18 hr culture of the host E. coli ATCC 11303 and subsequently add 0.1 mℓ of the phage lysate. Immediately pour the contents of the surface of a previously prepared LCM agar plate and gently rock the plate to spread the soft agar evenly. When the agar layer has solidified (allow about 20 min), invert the plates and incubate at 30 C for 18 hr. Bacterial colonies which grow up on the plate are T4 resistant. Pick a colony using a needle and

transfer to a nutrient agar slant. Grow this culture and use this T4 resistant strain to seed your water sample.

Nutrient agar: Difco, petri plates each containing 15 mℓ of agar; 15 per student group.

LM broth.

Bactotryptone (Difco)	10 g
Yeast extract	5 g
$MgSO_4 \cdot 7H_2O$	0.25 g
NaCl	8 g
Distilled water	1000 mℓ

Adjust pH to 7.0 and sterilize at 121 C for 15 min. This broth is used to grow the seed E. coli. Dispense 50 mℓ in a 125 mℓ erlenmeyer flask prior to sterilization.

LCM broth: Same as LM broth with addition of $CaCl_2$ to a final concentration of 2.5×10^{-3} M. Add 10 mℓ sterile (autoclaved at 121 C for 15 min) $CaCl_2$ solution (27.8 g/ℓ) aseptically to each 1000 mℓ cool sterile LM broth. This medium is used for the growth of seed bacteriophage.

LM soft agar: Same as LM broth with the addition of 0.4% pure agar. Dispense 3 mℓ per 12 x 75 mm tube, plug and sterilize at 121 C for 15 min, 15 tubes per student group.

LCM agar: Same ingredients as LM broth with the addition of 1.0% agar. Autoclave at 121 C for 15 min. After sterilization, aseptically add 10 mℓ sterile $CaCl_2$ (27.8 g/ℓ) to each 1000 mℓ broth. Add to agar at 45 C. Prepare petri dishes each containing 15 mℓ of LCM agar, 15 per student group.

Standard dilution buffer: (See Experiment I-A-1).

EXPERIMENT III-E-2

REMOVAL OF BACTERIA BY WATER CHLORINATION

Purpose

To demonstrate the effectiveness of chlorine in drinking water disinfection and the effect of ammonia on the germicidal activity of chlorine.

References

1. White, G.C. "Disinfection: The Last Line of Defense for Potable Water," J. American Water Works Association, 67:410, 1975.
2. White, G.C. Handbook of Chlorination, Van Nostrand Reinhold Co., N.Y., pp. 182 and 301, 1972.

Introduction

Chlorine has been used to disinfect drinking water since the turn of the century. In many instances, especially for groundwater, chlorination is the sole water treatment process used. When chlorine-containing chemicals are added to water, reactions symbolized by the following equations for chlorine gas take place.

$$Cl_{2(g)} + H_2O \rightleftarrows HOCl + HCl$$

$$HOCl \rightleftarrows H^+ + OCl^-$$

$$HCl \rightarrow H^+ + Cl^-$$

The most effective disinfecting species in HOCl (it is approximately 80 times more effective than OCl^-). Because of the involvement of H^+ in the above equilibria it can be readily deduced that pH has a marked

effect on germicidal efficiency. Thus the percentage composition of a chlorine solution with respect to HOCl and OCl^- and pH is approximately as follows:

pH	% HOCl Present
4	100
5	99.7
6	96.8
7	75.2
8	23.3
9	2.9
10	0.3

Of the water quality parameters other than pH that affect the disinfection rate of chlorine, perhaps the most significant is the presence of ammonia and other nitrogen-containing materials. Generally stated, chlorine reacts with ammonia to form "combined chlorine" or chloramines of various types. The type of chloramine formed is a function of several factors including pH, the ratio of the concentration of ammonia and chlorine present and the reaction time. For example, at pH 7.0 monochloramines (NH_2Cl) are rapidly formed when the weight ratio of chlorine to ammonia-nitrogen is 5:1 or less. As more chlorine is added, dichloramines ($NHCl_2$) are formed particularly when the ratio of chlorine to ammonia-nitrogen reaches 10:1. Higher chlorine to ammonia-nitrogen ratios, up to 15:1, lead to the formation of nitrogen trichloride (NCl_3) and even higher ratios will allow the formation of free chlorine in solution and result in the oxidation of ammonia to products that may include N_2, N_2O and NO_3^-.

The formation of chloramines has a significant effect on the rate of disinfection. Thus monochloramine concentration may have to be approximately 25 times greater than a free available chlorine concentration to achieve the same rate of disinfection. Dichloramines are two to three times faster disinfectants than the monochloramines, but are still slower than free chlorine. The germicidal activity of trichloramine is not well understood.

In chlorination practice then, it is well to understand the impact of ammonia on disinfection rate. If mono- and di-chloramines are formed, contact times must be increased for effective disinfection.

In the presence of free available chlorine and at a neutral or acid pH, the rate of bacterial destruction by chlorine is quite rapid. For example at pH 7.0 and a temperature of 20 to 30 C, greater than 99% reduction of Escherichia coli can be achieved in less than 2 min at a free chlorine concentration of 0.05 mg/ℓ. At a pH of 8.5 at the same free chlorine concentration, it would take more than 20 minutes to achieve the same reduction in bacterial numbers.

Chlorination practice has been devoloped primarily around the destruction of enteric bacteria using E. Coli as the indicator organism. While such a method has been successful in its conservatism it has become evident through laboratory work conducted during the last decade that enteric viruses can be more resistant to chlorine than enteric bacteria. The question therefore arises as to whether coliform organisms are a conservative enough indicator of viral contamination and disinfection efficiency.

Procedure

A. Culture Preparation

1. Prepare an 18-24 hr culture of E. Coli as described in Experiment III-E-1. This should yield approximately 10^9 cells per mℓ of culture.

2. Centrifuge 10-20 mℓ of the E. coli culture at 7,000-10,000 XG for 20 min. Pour off the supernatant and resuspend the bacterial pellet in 10-20 mℓ sterile dilution buffer and centrifuge again as above. Pour off the supernatant and resuspend the bacteria in 10 mℓ sterile dilution buffer. This will be the inoculum used for the disinfection study.

3. Prepare a stock solution of lithium hypochlorite (LiOCl) by carefully weighing out 2.86 g of fresh powdered LiOCl containing 35% free available chlorine and dissolve in 1000 mℓ of distilled water.

4. The contact chambers to be used in this exercise are 3 sterile 250 mℓ erlenmeyer flasks. If available, trypsinizing flasks of the same volume would be preferable because of better mixing characteristics. To one of these flasks, containing a magnetic mixing bar, add 100 mℓ sterile water buffered at pH 6.5 and set on a magnetic stirring device. Add 1 mℓ washed E. coli suspension and mix vigorously. Remove 1 mℓ of this mixture and add to 2 mℓ of sterile buffered water containing 100 mg/ℓ of sodium thiosulfate. Set this sample aside for subsequent bacterial analysis of the initial concentration of bacteria.

5. To a second flask add 97 mℓ, pH 6.5 sterile buffered water. Add 3 mℓ of a 1:1000 dilution of stock lithium hypochlorite solution to

give a final free available chlorine concentration of approximately 0.2 mg/ℓ in the bacterial suspension. The contents of the flask should be continuously and vigorously mixed. Rapidly add 1 mℓ of washed E. coli suspension and remove 1 mℓ of the mixture after 10 sec, 30 sec, 45 sec and 1 min. As each 1 mℓ sample is removed from the reaction chamber, it should be mixed immediately with 2 mℓ sterile buffered dilution water containing 100 mg/ℓ thiosulfate. Set aside these "neutralized" samples for bacterial analysis.

6. The third flask is used to demonstrate the disinfecting properties of chlorine combined with ammonia to form monochloramines. To 98 mℓ sterile buffer, add 0.5 mℓ of stock ammonium chloride solution (1g/1000 mℓ distilled water). This will give an ammomia nitrogen concentration of 1.32 mg/ℓ. Subsequently, add 1 mℓ of a 1:10 dilution of stock lithium hypochlorite solution and mix for 15 min to convert the free chlorine to monochloramine. At the end of this reaction time, add 1 mℓ washed E. coli suspension. While the flask contents are being vigorously mixed withdraw 1 mℓ samples as in Step 5 above and add to 2 mℓ of neutralizing solution.

7. The collected and neutralized samples should be analyzed using the pour plate method described in Experiment I-A-1, using nutrient agar as the growth medium. Dilutions for the initial concentration of bacteria should range from 10^{-5} to 10^{-8}; dilutions for the samples exposed to free and combined chlorine should range from 10^{0} to 10^{-3} for each sample taken.

8. Incubate at 35 C for 24-48 hr and count the number of colonies produced. Calculate the number of E. coli present per mℓ in the

reaction flask during the various sample times taking into account the dilutions introduced in the "neutralizing" step.

Apparatus

Erlenmeyer flasks or trypsinizing flasks: 250 mℓ; 3 per student group.

Magnetic stirrer: 1 per student group.

Magnetic stirring bars: 3 per student group.

Petri dishes: sterile; 36 per student group.

Dilution blanks: 99 mℓ sterile dilution buffer; 12 per student group.

Culture tubes: containing 2 mℓ sterile dilution water with 100 mg/ℓ sodium thiosulfate added; 10 per student group.

Pipettes: sterile.

Reagents

Lithium hypochlorite: 35% free available chlorine, Lithium Corporation of American.

Ammonium chloride solution: 1 g NH_4Cl per 1000 mℓ distilled water.

Buffered water for reaction mixture: prepare same as standard buffered dilution water Experiment I-A-1 except adjsuted to pH 6.5 with NaOH.

Neutralizing solution: add 0.1 g sodium thiosulfate ($Na_2S_2O_3$) to 1000 mℓ standard buffered dilution water. Dispense 2 mℓ small culture tube, plug and sterilize.

Standard dilution buffer: See Experiment I-A-1.

Nutrient agar: Difco, dispense 15 mℓ per 16 x 1000 mm screw cap tube, sterilize and hold, melted, at 45-46 C; 36 tubes per student group.

APPENDIX I

CARE AND USE OF MICROSCOPE

Illumination

Illumination by artificial light is superior to daylight for studying fine details. Place the lamp about 1 ft from the microscope and direct the light beam to strike the mirror. Adjust the light source so that a bright spot is focused on the mirror. Adjust the plane side of the mirror to illuminate the specimen. A piece of ground glass is provided to interpose between the filament and the mirror to control the intensity of illumination.

Focusing

Raise the objectives using the coarse adjustment knob. Select a well-stained specimen and place on the stage and center it over the condenser.

Low-Power Objective (X10): Move the low power objective into position and lower it over the specimen. Focus by raising the objectives with the coarse control and further define the image using the fine controls. Adjust the microscope condenser to its proper vertical position by moving the condenser so that the ground glass is in focus with the specimen and lower it slightly. Control the intensity of illumination using the iris diaphragm.

After completing the above steps remove an eyepiece and look down the draw tube. A uniform circle of light will be seen when the light source, mirror and condenser have been adjusted properly.

The iris diaphragm, when manipulated, should be sharply defined. If unevenly illuminated, adjust the mirror for full illumination.

Replace the eyepiece.

Binocular eyepieces are adjustable to fit the observer's eyespacing. Persons wearing glasses should be careful to prevent their lenses or the top lenses of the eyepiece from becoming scratched.

Medium-Power (high-Dry) Objective (X43): Unless the objectives are parfocal (i.e. when the nosepiece is rotated to the next object the specimen remains in focus) raise the objectives about 1 cm using the coarse focus and rotate the nosepiece until the objective clicks into place. Even with parfocal microscopes, thick specimen slides and individual differences in observers may require that the objectives be raised upon changing them.

Next place the eye on a level with the top of the specimen slide and lower the objective slowly using the coarse adjustment until it is only just clear of the slide. Do not touch the slide with the objective. Looking through the eyepiece, focus upwards slowly until the objective is in focus. Complete the focusing with the fine adjustment.

If the specimen is not in focus (the upward movement was too rapid) repeat the initial steps. Do not ever try to bring the specimen into focus by focusing downwards, when you are looking through the eyepiece.

Oil-Immersion Objective (X97): The procedures in focusing a specimen using the oil immersion objective are similar to those employed with the High-Dry objective except that a drop of oil is placed between the top of the specimen and bottom lens to increase the resolution.

First, raise the objective until it is about 1 cm above the slide.

Rotate the nosepiece to bring the oil immersion objective into position. Add a small drop of immersion oil to the center of the specimen slide. Place your eye level with the top of the specimen slide and lower the objective carefully until it makes contact with the oil. The working distance of oil immersion objectives is extremely small so that initial attempts at focusing may be difficult. Lower the objective further without touching the slide. Looking through the eyepiece, focus upwards with the fine adjustment. If the oil film breaks or if the objective is raised more than 2-3 mm above the slide without finding the specimen, repeat the initial steps. Adjust the mirror and iris diaphragm for optimum lighting when the specimen has been located and focused.

Cleaning

Lens paper is to be used for cleaning the eyepiece and objective lens. Other than removing the eyepiece for the initial illumination adjustment, no dismantling of the microscope body and lenses is needed.

At the beginning of each class period in which the microscope is to be used, wipe the lenses with lens paper. The best procedure is initially to softly brush the lens surface with the paper to remove any large particles which might scratch the lens. Next wipe off any accumulated oil. After each period of use wipe off the lenses in the same way. Xylene may be used to remove dried immersion oil from the 97X objective as well as any spills on the microscope stage. However, xylene should be used only sparingly because it may dissolve adhesives used to hold objective lens in place.

Storage

After cleaning the microscope, swing the low power objective into position and lower the objectives. Cover the microscope and return it to a proper storage compartment.

Calibration of the Microscope

Measurement of microscopic objects can be performed using an ocular micrometer. The ocular micrometer is a glass disc marked by a scale of etched lines. It is inserted into the eyepiece after unscrewing the top lens. Eyepieces permanently provided with a scale for measurement can be obtained and used also for this purpose. These are however, undesirable, as the scale in an eyepiece interferes with routine observations.

Calibrate the ocular scale as follows: place a stage micrometer on the microscope and focus on it, starting with the low power objective. Calibrate each objective separately. The scale of the stage micrometer is generally 2 mm long. This distance is divided into 20 parts and the distance between these lines is 100 µm, The first two divisions on the stage micrometer are divided into 20 segments each being 10 µm. To calibrate the ocular micrometer, find the number of ocular micrometer lines which correspond most closely to a number of lines on the stage micrometer; for example, it may be found that 19 divisions of the ocular micrometer correspond to 3 divisions of the stage micrometer. Each division on this part of the stage micrometer represents 100 µm; so 24 ocular divisions make 300 µm and each ocular division must equal 12.5 µm. With the high magnification objectives, the etched stage micrometer lines appear so thick that it is essential to use only one edge of these lines in setting the ocular micrometer scale.

In calibrating each lens combination, use the average of three independent measurements and carry out the calibration to the first decimal place.

An ocular scale so calibrated can be used directly in measurement. An organism on the stage may, for example, cover 10 ocular micrometer lines. Using the calibration described above, the distance between two lines is 12.5 μm and the length of the specimen would be 125 μm.

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